AWARD NUMBER: **W81XWH-11-1-0497**

TITLE: **Breast Cancer Stem Cells in Antiestrogen Resistance**

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REPORT DATE: **October 2014**

TYPE OF REPORT: **Final**

PREPARED FOR: **U.S. Army Medical Research and Materiel Command**
Fort Detrick, Maryland, 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2014	Final	1Aug2011 - 31Jul2014

4. TITLE AND SUBTITLE
Breast Cancer Stem Cells in Antiestrogen Resistance

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0497

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Creighton University
2500 California Plaza
Omaha, NE 68178

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our research program is to understand the function and underlying mechanisms of a novel estrogen receptor variant, ER-α36, in antiestrogen resistance of breast cancer stem/progenitor cells. In the whole funding period, we have demonstrated the stem/progenitor cells enriched from antiestrogen sensitive ER-positive breast cancer cells are refractory to and even stimulated by antiestrogens. The effects of antiestrogens on the ER-positive breast cancer stem/progenitor involve changes of both proliferation and differentiation. We also found that ER-α36 plays an important role in positive regulation of both ER-positive and –negative breast cancer stem/progenitor cells and contributes to the resistance of breast cancer stem/progenitor cells to antiestrogens presumably through mediating agonist activities of antiestrogens. Finally, we discovered novel regulatory loops of ER-α36 and EGFR/HER2 that play an important role in antiestrogen resistance and disruption of these loops sensitizes breast cancer stem/progenitor cells to antiestrogen. Thus, our study of the role and underlying mechanisms of breast cancer stem/progenitor cells in antiestrogen resistance not only provided important information about the function of breast cancer stem/progenitor cells in development of antiestrogen resistance, but also laid the foundation for development of novel therapeutic approaches to overcome antiestrogen resistance.

15. SUBJECT TERMS
None Listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	108	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Our research program is to study the role and underlying mechanisms of breast cancer stem/progenitor cells in antiestrogen resistance. One central subject of this study is to understand the biological significance and underlying mechanisms of a novel estrogen receptor variant, ER-α36, in resistance of breast cancer stem/progenitor cells to antiestrogens. We have demonstrated that ER-α36-mediated rapid estrogen signaling plays an important role in maintenance and positive regulation of breast cancer stem/progenitor cells. We found that antiestrogen resistant ER-positive breast cancer cells contain high populations of stem/progenitor cells, and the stem/progenitor cells enriched from antiestrogen sensitive ER-positive breast cancer cells are refractory to and even stimulated by antiestrogens. The effects of antiestrogens on the ER-positive breast cancer stem/progenitor involve changes of both proliferation and differentiation. We also found that ER-α36 contributes to the resistance of breast cancer stem/progenitor cells to antiestrogens presumably through mediating agonist activities of antiestrogens. Finally, we discovered the existence positively regulatory loops between ER-α36 and EGFR/HER2 in breast cancer stem/progenitor cells from both ER-positive and –negative breast cancer cells that paly important roles in development of antiestrogen resistance, and disruption of these regulatory loops sensitizesbreast cancer stem/progenitor cells to antiestrogens. Thus, we have accomplished more works thanwhat wasproposed in the original grant, which resulted in publications ofsix manuscripts, one manuscript submitted and one in preparation. Our study of the role and underlying mechanisms of breast cancer stem/progenitor cells in antiestrogen resistance not only provided important information about the function of breast cancer stem/progenitor cells in development of antiestrogen resistance, but also laid the foundation for development of novel therapeutic approaches to interfere with antiestrogen resistance.

BODY

Task 1: To determine whether the breast cancer stem/progenitor cells from ER-positive breast cancer cells are involved in antiestrogen resistance and the function of ER-α36 in the resistance of ER-positive breast cancer stem/progenitor cells to antiestrogens.

Antiestrogens such as tamoxifen provided a successful treatment for ER-positive breast cancer for the past four decades. However, most breast tumors are eventually resistant to tamoxifen therapy. The molecular mechanisms underlying tamoxifen resistance have not been well established. In this grant, we decided to first investigate the possible involvement of ER-α36 in tamoxifen resistance. We found that tamoxifeninduced ER-α36 expression， and tamoxifen-resistant MCF7 cells expressed high levels of endogenous ER-α36. In addition, MCF7 cells with forced expression of recombinant ER-α36 and H3396 cells expressing high level of endogenous ER-α36 were resistant to tamoxifen (Zhang et al., 2013). Knockdown of ER-α36 expression in tamoxifen-resistant breast cancer cells with the shRNA method restored tamoxifen sensitivity, andtamoxifen acted as a potent agonist by activating phosphorylation of the AKT kinase in ER-α36 expressing cells. Finally, we found that cells with high levels of ER-α36 expression were hypersensitive to estrogen; activating ERK phosphorylation at pM range (Zhang et al., 2013). Our results thus demonstrated that enhanced ER-α36 expression is one of the mechanisms by which ER-positive breast cancer cells acquiretamoxifenresistance.

Extensive researches were conducted to understand the molecular pathways involved in antiestrogen resistance and have revealed that multiple signaling molecules and pathways such as EGFR and HER2 play important roles in antiestrogen resistance. All of these pathways often bypass the requirement of estrogen signaling for growth of ER-positive breast cancer cells.

Previously, we reported the existence of positive regulatory loops between ER-α36 and EGFR/HER2 in ER-negative breast cancer cells (Zhang et al., 2011; Kang et al., 2012. In triple-negative breast cancer MDA-MB-231 and MDA-MB-436 cells, knockdown of ER-α36 expression enhances EGFR protein degradation through the proteasome system while EGFR signaling pathway up-regulates the promoter activity of ER-α36 through an Ap1 binding site in the 5' flanking sequence of ER-α36 gene (Zhang et al., 2011). In HER2 overexpressing breast cancer SKBR3 cells, ER-α36-mediated signaling positively regulates HER2 transcription while HER2 signaling up-regulates the promoter activity of ER-α36. Thus, we sought to studythe possible involvement of these regulatory loops in development of tamoxifen resistance in ER-positive breast cancer cells. Recently, we reported that tamoxifen treatment induced expression of ER-α36, EGFR and HER2 in ER-positive breast cancer cells through the ER-α36-EGFR/HER2 positive regulatory loops and these regulatory loops play important roles in antiestrogen resistance of ER-positive breast cancer cells (Yin et al., 2014).

Currently, it is well known thatthe breast cancer stem cells (BCSCs) play important roles in breast cancer occurrence, recurrence and metastasis. However, the role of estrogen signaling, a signaling pathway important in development and progression of breast cancer, in regulation of BCSC has not been well established. Thus, we also investigated the function and the underlying mechanism of ER-α36-mediated rapid estrogen signaling in growth regulation of the ER-positive breast cancer stem/progenitor cells.

We reported that ER-positive breast cancer stem/progenitor cells express high levels of ER-α36. 17-β-estradiol (E2β) treatment increased the population of ER-positive breast cancer stem/progenitor cells while failed to do so in the cells with knocked-down levels of ER-α36 expression (Deng et al., 2013). ER-positive breast cancer cells with forced expression of recombinant ER-α36, however, responded strongly to E2β treatment by increasing growth of the stem/progenitor cells*in vitro* and tumor-seeding efficiency *in vivo*. We also found that E2β treatment stimulated proliferation of the progenitor cells through ER-α36-mediated rapid estrogen signaling pathway (Deng et al., 2013).Our results thus demonstrated thatER-α36-mediated rapid estrogen signaling plays an important role in positive regulation and maintenance of ER-positive breast cancer stem/progenitor cells.

We then investigated the roles of breast cancer stem/progenitor cells in antiestrogen resistance as well as the underlying molecular mechanisms.We used ER-positive breast cancer cells MCF7 and T47D as well as variants with different levels of ER-α36 expression as model systems. The effects of antiestrogenstamoxifen and ICI 182, 780 on breast cancer stem/progenitor cells' ability of growth, self-renewal, differentiation and tumor seeding were examined using tumorsphere formation, flowcytometry, indirect immunofluorences and *in vivo*xenograft assays.

We found that the cancer stem/progenitor cells enriched from ER-positive breast cancer cells were more resistant to antiestrogens than the bulk cells (Deng et al., 2014). Antiestrogens increased the percentages of the stem/progenitor cells from ER-positive breast cancer cells through stimulation of luminal epithelial lineage specific ER-positive breast cancer progenitor cellswhile failed to do so in the cells with knocked-down levels of ER-α36 expression (Deng et al., 2014). Antiestrogen treatment enriched the breast cancer stem/progenitor cells from ER-positive breast cancer cells (Deng et al., 2014). We also found that antiestrogens acted as agonists to induce the PI3K/AKT signaling in breast cancer stem/progenitor cells (Deng et al., 2014). These results strongly indicated that ER-α36-mediated PI3K/AKT signaling induced by

antiestrogens is one of the mechanisms by which the breast cancer stem/progenitor cells that express high levels of ER-α36 become antiestrogen resistant.

Finally, we found that antiestrogen resistant cells expressing high levels of growth factor receptor HER-2 such as BT474 and MCF7/HER-2/18 contain high populations of stem/progenitor cells and express high levels of ER-α36 (Yin et al., submitted). A positive regulatory loop between ER-α36 and HER2 plays an important role in antiestrogen resistance of the breast cancer stem/progenitor cells from HER2 expressing cells (Yin et al., submitted). These results provided further evidence to support the hypothesis that breast cancer stem/progenitor cells are involved inantiestrogen resistance, and increased populations of breast cancer stem/progenitor cells is a novel and important mechanism underlying development of antiestrogen resistance.

Task 2: To investigate the function and the underlying mechanisms of ER-α36 in antiestrogen resistance of the ER-negative breast cancer stem/progenitor cells.

ER-α36 plays an important role in maintenance of the stem/progenitor cells from ER-negative breast cancer SKBR3 cells. Knockdown of ER-α36 expression in SKBR3 cells dramatically reduced the population of ALDH positive stem/progenitor cells (Kang et al., 2013). We also established stable cells withknocked-down levels of ER-α36 expression in triple-negative breast cancer MDA-MB-231 and MDA-MB-436 cells **(**Zhang et al., 2011). The cells withknocked-down levels of ER-α36 expression also expressed down-regulated levels of EGFR protein**(**Yin et al., in preparation), consistent with our previous report that there is a positive regulatory loop between ER-α36 and EGFR expression that fuels the malignant growth of triple-negative breast cancer (Zhang *et al.,* 2011). The cells with knocked-down levels of ER-α36 expression failed to form tumors in nude mice (Zhang et al., 2011), suggesting the ER-negative breast cancer cells with knocked-down levels of ER−α36 expression contain less tumor initiating cells i.e. cancer stem/progenitor cells.

To examinethe effects of tamoxifen on the stem/progenitor cells enriched from the ER-negative breast cancer cells with knocked-down levels of ER-α36 expression, we employed tumorsphere formation assays using MDA-MB-436 and MDA-MB-231 cells transfected with the ER-α36 shRNA expression vector. Cells were plated at low density (5,000 viable cells/well) in 6-well ultra-low attachment plates for seven days in the absence and presence of different concentrations of tamoxifen. We found that ER-negative breast cancer cells as well as ER-negative breast cancer stem/progenitor cells with knocked-down levels of ER-α36 became sensitive to tamoxifen (Yin et al., in preparation). This result thus indicated that ER-α36 is involved in antiestrogen resistance of ER-negative breast cancer stem/progenitor cells.

We also examined the effects of antiestrogens on differentiation of ER-negative breast cancer stem cells.Tumorspheres formed by ER-positive breast cancer MDA-MB-231 and SKBR3 cells were treated with vehicle, estrogen (E2β) or antiestrogensfor five days, and examined with indirect immunofluoresces assay to determine differentiation lineages of these cells using cytokeratin 18 (CK18) for epithelial cells, CD10 for myoepithelial cells and Vimentin for mesenchymal cells. We found thatantiestrogen treatmentdecreased the number of cells expressing CK18 or CD10 in MDA-MB-231 and SKBR3 cells whilefailed to influence vimentin expressing cells (Yin et al., in preparation). Our results suggested that antiestrogensmightattenuate differentiation of ER-negative breast cancer stem cells.

Based on the importance of ER-α36-mediated signaling in maintenance and regulation of cancer stem/progenitor cells in both ER-positive and –negative breast cancer, we hypothesized that ER-α36 may serve as a target to develop novel therapeutic agents to eradicate breast cancer stem/progenitor cells. Wehave discovered that several flavonoid derivatives purified from the bark of the Paper Mulberry tree (*Broussonetiapapyrifera*) (L.) were able to down-regulate ER-α36 expression (Guo et al., 2013a). We then examined the growth inhibitory activity of the most potent ER-α36 down-regulator Broussoflavonol B in the breast cancer stem/progenitor cells derived from ER-negative breast cancer SK-BR-3 and MDA-MB-231 cells. We found that Broussoflavonol B, as a potent inhibitor of ER-α36 expression, attenuated growth of ER-negative breast cancer stem/progenitor cells and also induced differentiation of these cells (Guo et al., 2013 b & c).These results provided strong evidence for the concept that ER-α36 can serve as a target to develop novel and effective therapeutic approaches to "de-stem" breast cancer stem cells.

It is well established that gained expression of the EGFR and HER2 is one of the mechanisms underlying antiestrogen resistance. However, the mechanism by which ER-positive breast cancer cells gain expression of EGFR and HER2 is unknown. Recently, we reported that tamoxifeninduced expression of ER-α36, EGFR and HER2 in tamoxifen sensitive MCF7, T47D and H3396 cells (Yin et al., 2014). In addition, tamoxifen resistant MCF7 cells established by long-term cultivation in the presence of tamoxifen also expressed elevated levels of endogenous ER-α36, EGFR and HER2 (Yin et al., 2014). Knockdown of ER-α36 expression in tamoxifen resistant MCF7 cells reduced EGFR and HER2 expression, and MCF7 cells with forced expression of ER-α36 expressed increased levels of EGFR and HER2. Inhibition of both EGFR and HER2 signaling pathways with the dual kinase inhibitor Lapatinib down-regulated ER-α36 expression (Yin et al., 2014).Taken together, our results indicated that the ER-α36-EGFR/HER2 positive regulatory loops are one of the underlying mechanisms of ER-positive breast cancer cells gained expression of the growth factor receptors during antiestrogen treatment.

In addition, the caner stem/progenitor cells enriched from ER-positive breast cancer cells express enhanced levels of ER-α36, EGFR and HER2. These positive regulatory loops contribute to antiestrogen resistance of breast cancer stem/progenitor cells. We decided to study if disruption of these regulatory loops sensitizes breast cancer stem/progenitor cells to antiestrogens.Recently, we reported that Lapatinib inhibited phosphorylation of both EGFR and HER2 and down-regulated ER-α36 in breast cancer stem/progenitor cells enriched from ER-positive breast cancer cells (Yin et al., 2014). In addition, ER-α36 down-regulator Broussoflavonol B also down-regulated expression levels of EGFR and HER2 in ER-positive breast cancer stem/progenitor cells (Yin et al., 2014). These results indicated that both the dual kinase inhibitor Lapatinib and the ER-α36 down-regulator Broussoflavonol B disrupt the ER-α36-EGFR/HER2 positive regulatory loops and down-regulated expression levels of ER-α36, EGFR and HER2.Importantly, disruption of the ER-α36-EGFR/HER2 positive regulatory loops restored tamoxifen sensitivity in breast cancer stem/progenitor cells (Yin et al., 2014). Similar effects of Lapatinib and Broussoflavonol B were also observed in breast cancer stem/progenitor cells from HER2 expressing cells and ER-negative breast cancer cells (Yin et al., submitted and in preparation). Our results thus, for the first time, revealed the existence of the ER-α36-EGFR/HER2 positive regulatory loops in antiestrogen resistance of breast cancer stem/progenitor cells from both ER-positive and –negative breast cancer cells and provided

strong rationales for development of novel therapeutic approaches to treat antiestrogen resistant breast cancer by targeting the ER-α36-EGFR/HER2 positive regulatory loops.

KEY RESEARCH ACCOMPLISHMENTS

1. We demonstrated that antiestrogen-resistant as well as HER-2 expressing ER-positive breast cancer cells contain high populations of stem/progenitor cells.
2. We found that the stem/progenitor cells enriched from antiestrogen sensitive ER-positive breast cancer cells are refractory to and even stimulated by antiestrogens.
3. We found that antiestrogens influence both proliferation and differentiation of the ER-positive breast cancer stem/progenitor cells.
4. We also found that a novel estrogen receptor variant, ER-α36, plays an important role in positive regulation of both ER-positive and –negative breast cancer stem/progenitor cells and contributes to the resistance of breast cancer stem/progenitor cells to antiestrogens presumably through mediating agonist activities of antiestrogens.
5. We discovered novel regulatory loops between ER-α36 and EGFR/HER2 that play important role in antiestrogen resistance of breast cancer stem/progenitor cells.
6. We also discovered that disruption of these positive regulatory loops sensitizes breast cancer stem/progenitor cells to antiestrogens.

REPORTABLE OUTCOMES

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Yin, L., Zhang, X. T., Guo, Y. M., Wang, Z. Y."The Dual Kinase Inhibitor LapanitibSensitizes ER-negative Breast Cancer Cells toTamoxifen". *In Preparation.*

CONCLUSIONS

Since mitogenic estrogen signaling plays a pivotal role in development and progression of ER-positive breast cancer, treatment with antiestrogens such as tamoxifen and fulvetrant (ICI 182, 780)provides a successful treatment option for ER-positive breast cancer patients in the past four decades. However, despite the significant anti-neoplastic activity of antiestrogens, most breast tumors are eventually resistant to antiestrogen therapy, which largely affects the efficacy of antiestrogen treatment. The exact mechanisms underlying the antiestrogen resistance in these ER-positive tumors have not been well established. Several mechanisms have been postulated to be involved in the tamoxifen resistance such as increased growth factor signaling, metabolism of tamoxifen by CYP2D6 variants, altered expression of co-regulators, mutations of ER-α.

In this grant, we proposed to study the role and underlying mechanisms of breast cancer stem/progenitor cells in antiestrogen resistance. One central subject of this study is to understand the biological significance of a novel estrogen receptor variant, ER-α36, in resistance of breast cancer stem/progenitor cells to antiestrogens. Now, we have accomplished most works proposedin the grant.

We discovered that tamoxifeninduced ER-α36 expression and tamoxifen-resistant MCF7 cells expressed high levels of endogenous ER-α36 (Zhang et al., 2013). In addition, MCF7 cells with forced expression of recombinant ER-α36 and H3396 cells expressing high level of endogenous ER-α36 were resistant to tamoxifen (Zhang et al., 2013). Knockdown of ER-α36 expression in tamoxifen-resistant breast cancer cells with the shRNA method restored tamoxifen sensitivity, andtamoxifen acted as a potent agonist by activating phosphorylation of the AKT kinase in ER-α36 expressing cells (Zhang et al., 2013). These results demonstrated that ER-α36-mediated rapid antiestrogensignaling is involved in development of antiestrogen resistance.

Wealso found that cells with high levels of ER-α36 expression were hypersensitive to estrogen; activating ERK phosphorylation at pM range (Zhang et al., 2013). Previously, it was reported that long-term estrogen deprivation with hormonal therapy resulted in "adaptive" changes in breast cancer cells; making these cells hypersensitive to estrogen (Masamura et al., 1994; Santen et al., 2004), suggesting the expression levels of ER-α36 is elevated in response to low concentrations of estrogen during antiestrogen treatment. Thus, our results indicated that gained expression of ER-α36 is one of the "adaptive" changes in breast cancer cells after a long-term estrogen deprivation resulted from antiestrogen treatment.

We discovered that antiestrogen resistant ER-positive breast cancer cells such as ER-positive breast cancer MCF7 cells selected through long-term culture of cells in the presence of tamoxifen, and cells expressing high levels of growth factor receptor HER-2 such as BT474 and MCF7/HER-2/18 contain high populations of stem/progenitor cells (Yin et al., 2014 & submitted). These results provided evidence to support the hypothesis that breast cancer stem/progenitor cells are involved in antiestrogen resistance, and increased populations of breast cancer stem/progenitor cells is a novel and important mechanism underlying development of antiestrogen resistance.

We further demonstrated that the stem/progenitor cells enriched from antiestrogen sensitive ER-positive breast cancer cells were refractory to antiestrogens tamoxifen and fulvetrant. Antiestrogens tamoxifen and fulvestrant at low concentrations (< 1μM) even stimulated

proliferation of the stem/progenitor cells (Deng et al., 2014). Immunofluorescence staining showed that both tamoxifen and fulvetrant treatment increased the number of ALDH1 positive cells, suggesting an increase of the population of the breast cancer stem/progenitor cells (Deng et al., 2014). Tamoxifen treatment also increased the number of CK18 positive cells while fulvestrant decreased the number of CK18 positive cells, suggesting that tamoxifen may stimulate the lineage-specific progenitor cells and fulvestrant may inhibit differentiation of breast cancer stem cells (Deng et al., 2014). To our knowledge, this is the first time to show that breast cancer stem/progenitor cells are resistant to antiestrogens and antiestrogens influence both proliferation and differentiation of breast cancer stem/progenitor cells. We further demonstrated antiestrogen treatment selects and enriches breast cancer stem/progenitor cells that are resistant to antiestrogens and exhibit strong tumor seeding efficiency (Deng et al., 2014). Taken together, our resultsindicate that antiestrogen therapy, while killing the bulk of breast tumor cells, may eventually fail since they do not eradicate breast cancer stem/progenitor cells that survive to regenerate new tumors.

We found that novel estrogen receptor variant, ER-α36, plays an important role in positive regulation of both ER-positive and –negative breast cancer stem/progenitor cells and is involved in the resistance of breast cancer stem/progenitor cells to antiestrogens, presumably through mediating agonist activities of antiestrogens.We also discovered novel regulatory loops between ER-α36 and EGFR/HER2 in breast cancer stem/progenitor cells that is critical for maintenance of breast cancer stem/progenitor cells and disruption of these loops with the dual kinase inhibitor Lapatinibsensitizes breast cancer stem/progenitor cells to antiestrogens.

With the support of this grant, we identified a natural compound, BroussoflavonolB, that was able to downregulate ER-α36 expression and to disrupt the positive regulatory loops between ER-α36 and EGFR/HER2. We also found Broussoflavonol B treatment inhibited growth of breast cancer stem/progenitor cells from ER-negative breast cancer cells (Guo et al., 2013b & c) and sensitized breast cancer stem/progenitor cells from tamoxifen resistant ER-positive breast cancer cells to tamoxifen (Yin et al., 2014). These findings are of both biological and clinical significance.

Many signaling pathways involved in regulation of normal stem cell fate, self-renewal, and maintenance including Hedgehog, Bmi-1, Wnt, NOTCH, HER-2, p53 and PTEN/Akt/β-catenin signaling have been identified to play roles in breast cancer stem/progenitor cell (Korkaya*et al.*, 2008 & 2009). However, the involvement of estrogen signaling, a major signaling pathway in breast cancer development, in regulation of breast cancer stem/progenitor cells has not been established, mainly because expression of estrogen receptor-α (ER-α) in breast cancer stem/progenitor cells remains controversial. It was reported that stem cells isolated from normal mammary gland and breast cancer tissues lack expression of the full-length ER-α (Sleeman *et al.*, 2006). Our results for the first time demonstrated that breast cancer stem/progenitor cells highly expressed ER-α36, a variant of ER-α and the rapid estrogen signaling mediated by ER-α36 plays an important role in positive regulation of breast cancer stem/progenitor cells. Our research also provided novel and important rationales to develop novel therapeutic approaches to eradicatebreast cancer stem/progenitor cells from both ER-positive and –negative breast cancer by targetingER-α36-mediated estrogen signaling.

The discovery of breast tumor cells that behave like stem cells and that are resistant to chemotherapy drugs, radiation therapy and antiestrogens provided a reasonable explanation for the difficulty to eradicate breast cancer. Novel mechanisms and targets for development of effective therapeutic approaches to inhibit growth of breast cancer stem/progenitor cells are

urgently needed. We found that the ER-α36 downregulatorBroussoflavonol Balso downregulated EGFR and HER2 expression and inhibited growth of breast cancer stem/progenitor cells from both ER-positive and –negative breast cancer cells. We also found that the dual kinase inhibitor Lapatinib not only inhibited EGFR and HER2 signaling but alsodownregulated ER-α36 expression, which sensitized breast cancer stem/progenitor cells to antiestrogentamoxifen. Thus our findings that disruption of the ER-α36 and EGFR/HER2 regulatory loops restores antiestrogen sensitivity in breast cancer stem/progenitor cellsprovided a rational to development of novel therapeutic approaches by targeting the ER-α36 and EGFR/HER2 regulatory loops in breast cancer stem/progenitor cells, which will ultimately revolutionize current therapeutic approaches.

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Endocrinology. First published ahead of print April 1, 2013 as doi:10.1210/en.2013-1116

Estrogen Receptor-α Variant, ER-α36, is Involved in Tamoxifen Resistance and Estrogen Hypersensitivity

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Antiestrogens such as tamoxifen provided a successful treatment for ER-positive breast cancer for the past two decades. However, most breast tumors are eventually resistant to tamoxifen therapy. The molecular mechanisms underlying tamoxifen resistance have not been well established. Recently, we reported that breast cancer patients with tumors expressing high concentrations of ER-α36, a variant of ER-α, benefited less from tamoxifen therapy than those with low concentrations of ER-α36, suggesting that increased ER-α36 concentration is one of the underlying mechanisms of tamoxifen resistance. Here, we investigated the function and underlying mechanism of ER-α36 in tamoxifen resistance. We found that tamoxifen increased ER-α36 concentrations and tamoxifen-resistant MCF7 cells expressed high concentrations of ER-α36. In addition, MCF7 cells with forced expression of recombinant ER-α36 and H3396 cells expressing high concentrations of endogenous ER-α36 were resistant to tamoxifen. ER-α36 downregulation in tamoxifen-resistant cells with the shRNA method restored tamoxifen sensitivity. We also found tamoxifen acted as a potent agonist by activating phosphorylation of the AKT kinase in ER-α36 expressing cells. Finally, we found that cells with high concentration of ER-α36 protein were hypersensitive to estrogen; activating ERK phosphorylation at pM range. Our results thus demonstrated that elevated ER-α36 concentration is one of the mechanisms by which ER-positive breast cancer cells escape tamoxifen therapy and provided a rational to develop novel therapeutic approaches for tamoxifen resistant patients by targeting ER-α36.

Since mitogenic estrogen signaling plays a pivotal role in development and progression of ER-positive breast cancer, treatment with antiestrogens such as tamoxifen (TAM) provides a successful option for ER-positive breast cancer patients in the past four decades. However, despite the significant antineoplastic activity of TAM, most breast tumors are eventually resistant to TAM therapy, which largely affects the efficacy of this treatment. Essentially, two forms of TAM resistance occur: de novo and acquired resistance (reviewed in 1–3). Although ER-α absence is the most common de novo resistance mechanism, about 50% ER-positive breast cancer patients with advanced disease do not respond to TAM treatment by the time of diagnosis (reviewed in 2). The exact mechanisms underlying the de novo TAM resistance in these ER-positive tumors are largely unknown. Several mechanisms have been postulated to be involved in the TAM resistance such as increased growth factor signaling, metabolism of TAM by CYP2D6 variants, altered expression of coregulators, mutations of ER-α (reviewed in 3, 4). In addition, most initially responsive breast tumors gradually acquire TAM resistance by loss of TAM responsiveness, the acquired resistance. The mechanisms by which breast tumors lose their TAM responsiveness have not been well established. Breast tumors with acquired TAM resistance frequently retain ER-α expression that would still classify them as ER-positive tumors (3). Therefore, loss of ER-α expression is not a major mechanism driving acquired TAM resistance. Another acquired TAM resistance phenotype has been described in breast cancer xenografts that exhibit a switch from a TAM-inhibitory phenotype to a TAM-stimulated one (5, 6). The agonist activity of TAM in this

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Received February 1, 2013. Accepted March 21, 2013.

Abbreviations:

model may be due to the enhanced growth factor signaling that is often associated with acquired TAM resistance (reviewed in 7). However, the molecular mechanism underlying this type of acquired TAM resistance has not been well established.

During development of acquired antiestrogen resistance, breast cancer cells usually undergo adaptive changes in response to inhibitory effects of antiestrogens (8). Adaptive changes also occur in response to aromatase inhibitor therapy in postmenopausal patients or from oophorectomy in premenopausal patients (9, 10). Using a MCF7 breast cancer model system, Santen's group demonstrated that deprivation of estrogen for a prolonged period of time confers these cells hypersensitive to low concentrations of estrogen (8–11). In these hypersensitive cells, 17-β-estradiol (E2) stimulates cell proliferation at pM range while the wild-type cells require nM range E2 to induce cell growth (11). However, the exact molecular events in the development of this "adaptive hypersensitivity" have not been elucidated although up-regulation and membrane localization of ER-α, activation of the nongenomic estrogen signaling, as well as induction of c-Myc and c-Myb have been proposed to be involved in this process (8, 12).

Previously, our laboratory identified and cloned a variant of ER-α, ER-α36, which has a molecular weight of 36-kDa (13, 14). The transcript of ER-α36 is initiated from a previously unidentified promoter in the first intron of the ER-α gene (15). This ER-α differs from the original 66 kDa ER-α (ER-α66) because it lacks both transcriptional activation domains (AF-1 and AF-2) but retains the DNA-binding and dimerization domains, and partial ligand-binding domain (13). ER-α36 is mainly localized near the plasma membrane and mediates membrane-initiated estrogen signaling (14). We also found that the breast cancer patients with tumors expressing high concentrations of ER-α36 benefited less from TAM therapy than those with low concentrations of ER-α36 (16), suggesting that increased ER-α36 concentration is one of the underlying mechanisms of TAM resistance. Recently, we also reported that ER-α36 is able to mediate agonist activity of TAM and ICI 182, 780 (17, 18) such as activation of the MAPK/ERK and the PI3K/AKT signaling pathways, indicating these antiestrogens may loss their growth inhibitory activities in cells with increased ER-α36 expression.

Based on these observations, we hypothesized that ER-α36 is involved in TAM resistance. Using ER-positive breast cancer MCF7 cells with different concentrations of ER-α36 as model systems, we investigated ER-α36 function in TAM resistance. Here, we present evidence to demonstrate that ER-α36 plays an important role in TAM resistance presumably through mediating agonist activity of TAM and estrogen hypersensitivity.

Materials and Methods

Chemicals and Antibodies

17β-estradiol (E2) was purchased from Sigma Chemical Co. (St. Louis, MO). Anti-phospho-p44/42 ERK (Thr202/Tyr204) (197G2) mouse monoclonal antibody (mAb), anti-p44/42 ERK (137F5) rabbit mAb, anti-phospho-AKT (Ser473) (D9E) rabbit mAb and anti-AKT (pan) (C67E7) Rabbit mAb, anti-EGFR and HER2 antibodies were purchased from Cell Signaling Technology (Boston, MA). Antibodies of ER-α66 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-ER-α36 antibody was generated by the custom service provided by the Pacific Immunology Corp. (Ramona, CA) using the last 20 amino acids of the ER-α36 encoded by the exon 9 that is unique to ER-α36 as an immunogen. The produced antibody was purified with an affinity column made of immunogen peptides. The antibody was characterized and validated with a number of experiments including immpunoprecipitation, immunofluorescence staining, Western blot analysis. The antibody specifically recognizes ER-α36 and does not cross react with ER-α66.

Cell culture and establishment of stable cell lines

The MCF7 cell line (ATCC) and its derivatives as well as H3396 cells (a kind gift from Dr. Leia Smith of Seattle Genetics) were maintained at 37°C in a 10% CO_2 atmosphere in IMEM without phenol red and 10% fetal calf serum. To establish stable cell lines with knocked-down concentrations of ER-α36, we constructed an ER-α36 specific shRNA expression vector by cloning the DNA oligonucleotides 5′-GATGCCAATAGGTACT-GAATTGATATCCGTTCAGTACCTATT GGCAT-3′ from the 3′UTR of ER-α36 gene into the pRNAT-U6.1/Neo expression vector from GenScript Corp. Briefly, cells transfected with the empty expression vector and ER-α36 shRNA expression vector were selected with 500 μg/ml G418 for three weeks, and more than 20 individual clones from transfected cells were pooled, examined for ER-α36 expression with Western blot analysis and retained for experiments. For ERK1/2 and AKT activation assays, cells were treated with vehicle (ethanol) and indicated concentrations of tamoxifen or E2.

To examine cell growth in the presence or absence of antiestrogens, cells maintained for three days in phenol red-free DMEM plus 2.5% dextran-charcoal-stripped fetal calf serum (HyClone, Logan, UT) were treated with different concentrations of tamoxifen, 17β-estradiol or ethanol vehicle as a control. The cells were seeded at 1×10^4 cells per dish in 60 mm dishes and the cell numbers were determined using the ADAM automatic cell counter (Digital Bio., Korea) after seven days. Five dishes were used for each treatment and experiments were repeated at least three times.

Western blot analysis

For immunoblot analysis, cells washed with PBS were lysed with the lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 0.25 mM EDTA pH8.0, 0.1% SDS, 1% Triton X-100, 50 mM

NaF) plus the protease and phosphatase inhibitors (Sigma). The protein amounts were measured using the DC protein assay kit (BIO-RAD Laboratories, Hercules, CA). The same amounts of the cell lysates were boiled for 5 min in loading buffer and separated on a SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane. The membranes were probed with various primary antibodies, HRP-conjugated secondary antibodies, and visualized with enhanced chemiluminescence (ECL) detection reagents (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). All Western blot experiments were performed at least three times. Band densities on developed films were measured and analyzed using Quantity One 1-D Analysis Software Version 4.6.7 (BIO-RAD Laboratories).

Statistical analysis

Data were summarized as the mean ± standard error (SE) using the GraphPad InStat software program. Tukey-Kramer Multiple Comparisons Test was also used, and the significance was accepted for $P < .05$.

Results

Tamoxifen treatment induces ER-α36 protein concentration in ER-positive breast cancer MCF7 cells

Previously, our laboratory identified and cloned a 36 kDa variant of ER-α, ER-α36 that functions differently from the 66 kDa full-length ER-α, ER-α66 (13, 14). Using an ER-α36 specific antibody, we further found that ER-α36 is highly expressed in established ER-negative breast cancer cells while weakly expressed in ER-positive breast cancer cells such as MCF7 (14). In order to investigate ER-α36 function in the activities of antiestrogens, we first examined whether TAM influences ER-α36 expression in MCF7 cells. The steady state concentration of ER-α36 protein in MCF7 cells treated with 1 μM of TAM for different time periods or different concentrations of TAM was examined with Western blot analysis. After TAM treatment, ER-α36 protein concentration was increased in MCF7 cells in a time and concentration dependent manner (Figure 1), indicating that TAM is able to increase ER-α36 concentration in ER-positive breast cancer MCF7 cells.

TAM-resistant ER-positive breast cancer MCF7 cells express high concentration of ER-a36 protein

To examine the possible involvement of ER-α36 in development of acquired TAM resistance, we cultured MCF7 cells in the presence of TAM (1 μM) for six months and pooled all surviving cells to establish a cell line MCF7/TAM. This cell line exhibited resistance to the growth inhibitory activity of TAM compared to the parental cells and TAM at 1 μM even acted as an agonist in MCF7/TAM cells (Figure 2A). Western blot analysis revealed that MCF7/TAM cells expressed higher concentration of ER-α36 protein compared to the MCF7 parental cells, while ER-α66 protein concentration was without significant change (Figure 2B), suggesting that MCF7 cells gained ER-α36 expression during development of acquired TAM

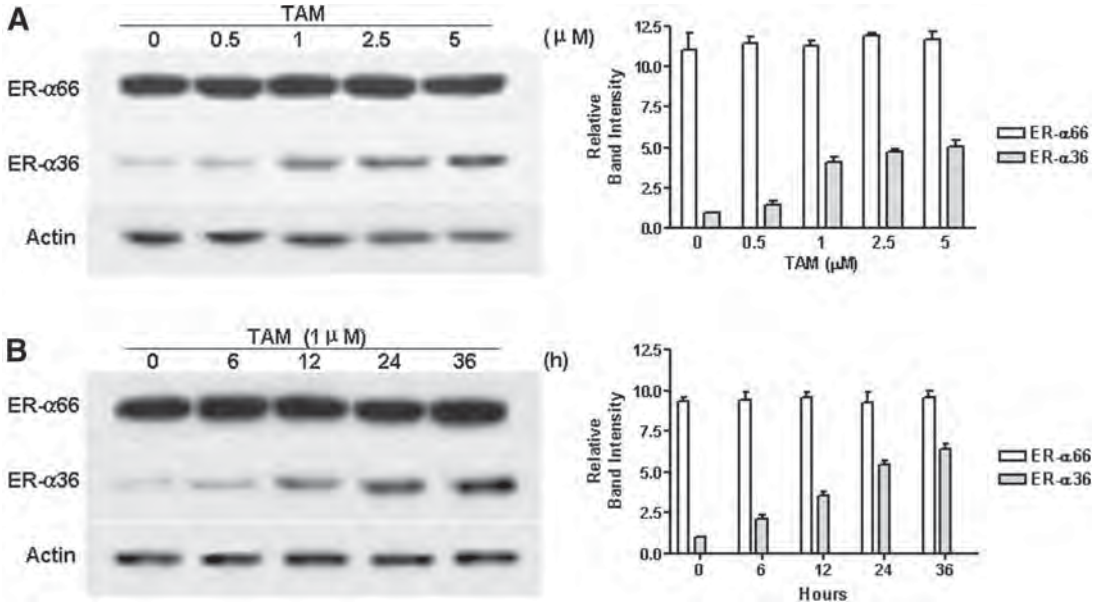

Figure 1. Tamoxifen treatment increases concentrations of ER-α36 protein. A. Western blot analysis of the concentrations of ER-α36 and 66 proteins in ER-positive breast cancer MCF7 cells treated with indicated concentrations of tamoxifen (TAM) for 12 h. B. Western blot analysis of MCF7 cells treated with 1 μM of TAM for indicated hours (h). All experiments were done at least three times and representative results are shown. Relative band intensities were obtained by quantitative densitometric scanning of autoradiographc signals and are shown with the ER-α36 band densities from the cells treated with vehicle (ethanol, 0 μM of TAM) or at 0 h that were arbitrarily set as 1. The columns represent the means of three experiments; bars, SE.

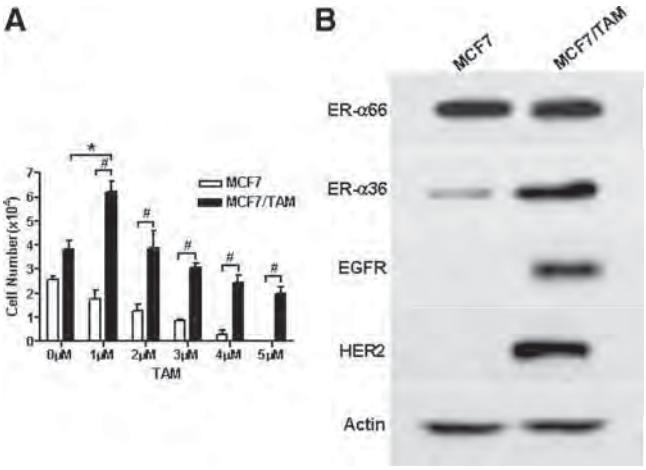

Figure 2. Tamoxifen resistant ER-positive breast cancer MCF7 cells express high concentrations of endogenous ER-α36. A. ER-positive breast cancer MCF7 cells and tamoxifen resistant MCF7 cells (MCF7/TAM) cells were treated with indicated concentrations of tamoxifen (TAM) for seven days and surviving cells were counted. The columns represent the means of three experiments; bars, SE. *, $P < .05$ for MCF/TAM cells treated with vehicle vs cells treated with 1 μM of TAM. #, $P < .01$ for MCF/TAM cells vs MCF cells treated with indicated concentrations of TAM. B. Western blot analysis of the expression concentrations of ER-α36 and 66, EGFR and HER2 in MCF7 and MCF7/TAM cells.

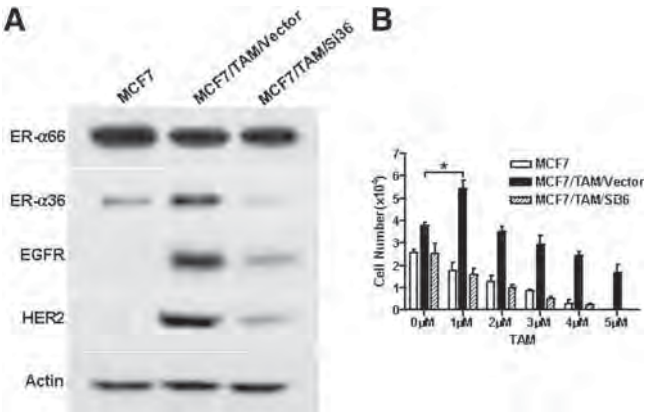

Figure 3. ER-α36 is involved in tamoxifen resistance. A. Western blot analysis of the concentrations of ER-α36 and 66, EGFR and HER2 proteins in MCF7 cells, MCF7/TAM cells transfected with the empty expression vector (MCF/TAM/Vector) and MCF7/TAM cells transfected with an ER-α36 specific shRNA expression vector (MCF7/TAM/Si36). B. Cells were treated with indicated concentrations of tamoxifen (TAM) for seven days and the numbers of surviving cells were determined. The columns represent the means of three experiments; bars, SE. *, $P < .05$ for MCF/TAM/Vector cells treated with vehicle vs cells treated with 1 μM of TAM.

resistance. MCF/TAM cells also expressed increased concentrations of EGFR/ErbB1 and HER2/ErbB2 proteins (Figure 2B), indicating MCF7/TAM cells also gained expression and signaling of the EGFR/HER2 pathway.

High concentration of ER-α36 protein confer TAM resistance

To confirm that elevated concentration of ER-α36 protein is involved in TAM resistance, we sought to down-regulate ER-α36 expression in MCF7/TAM cells using the shRNA approach. We established a cell line with knocked-down concentration of ER-α36 protein (MCF7/TAM/Si36) from MCF7/TAM cells using the shRNA method as evidenced by Western blot analysis (Figure 3A). We also noticed that the concentrations of EGFR and HER2 were also decreased in MCF7/TAM/Si36 cells (Figure 3A). ER-α36 knockdown restored the sensitivity of MCF7/TAM cells to the growth inhibitory effects of TAM to a level similar to parental MCF7 cells (Figure 3B). Our data thus suggested that elevated ER-α36 concentration is involved in development of acquired TAM resistance.

To further confirm elevated ER-α36 expression contributes to TAM resistance, we introduced recombinant ER-α36 into MCF7 cells that express high concentration of ER-α66 protein but lower concentration of ER-α36 to establish a stable cell line, MCF7/ER36. Western blot analysis confirmed that recombinant ER-α36 protein was highly expressed in MCF7/ER36 cells compared to the control MCF7 cells transfected with the empty expression vector (Figure 4A). We also observed that the EGFR expression was strongly increased while the HER2 expression was weakly increased in MCF7/ER36 cells (Figure 4A), consistent with our previous report that ER-α36 stabilizes EGFR protein (19). When MCF7/ER36 cells were treated with different concentrations of tamoxifen, these cells are more resistant to the growth inhibitory effects of TAM compared to the control MCF7 cells (Figure 4B), indicating that increased ER-α36 concentration is one of the underlying mechanism of tamoxifen resistance. We also found a breast cancer cell line H3396 that expressed high concentration of endogenous ER-α36 protein (Figure

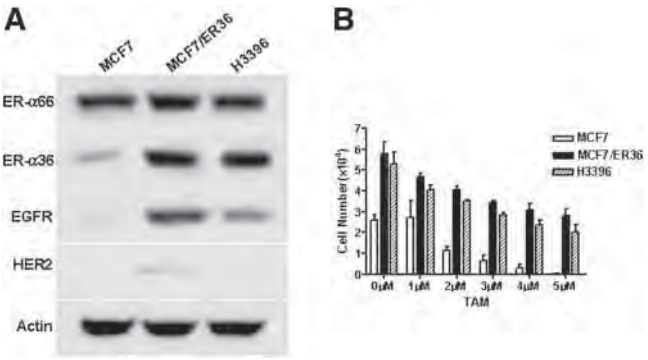

Figure 4. ER-positive breast cancer cells with elevated concentrations of ER-α36 protein are resistant to tamoxifen. A. Western blot analysis of the lysates from ER-positive breast cancer MCF7 cells, MCF7 cells with forced expression of ER-α36 (MCF7/ER36) and H3396 cells with high concentrations of endogenous ER-α36 protein. The concentrations of EGFR and HER2 proteins are also shown. B. Cells were treated with indicated concentrations of tamoxifen (TAM) for seven days and the numbers of surviving cells were counted. The columns represent the means of three experiments; bars, SE.

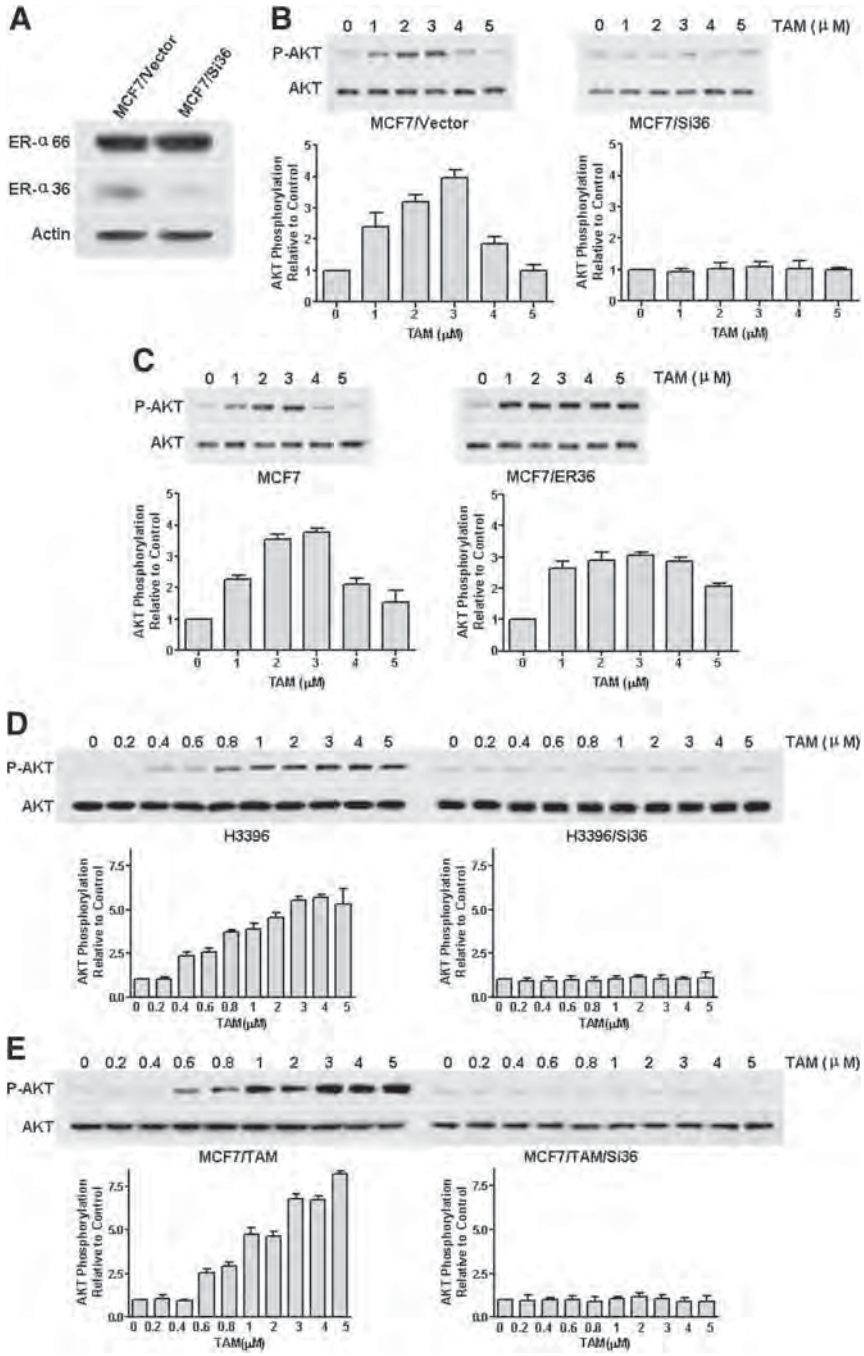

Figure 5. ER-α36 mediates tamoxifen-induced phosphorylation of the AKT kinase in ER-positive breast cancer MCF7 cells. A. Western blot analysis of ER-α36 and 66 protein concentrations in MCF7 cells transfected with an empty expression vector (MCF7/Vector) and MCF7 cells transfected with the ER-α36 specific shRNA expression vector (MCF7/Si36). B. Western blot analysis of phosphorylation of AKT in MCF7/Vector and MCF7/Si36 cells treated with indicated concentrations of tamoxifen (TAM) using phospho-specific or nonspecific AKT antibodies. C. Western blot analysis of phosphorylation of AKT in MCF7 and MCF7/ER36 cells treated with different concentrations of TAM. D. Western blot analysis of phosphorylation of AKT in H3396 and H3396/Si36 cells treated with indicated concentrations of TAM using phospho-specific or nonspecific AKT antibodies. D. Western blot analysis of phosphorylation of AKT in MCF7/TAM and MCF7/TAM/Si36 cells treated with indicated concentrations of TAM. All experiments were done at least three times and representative results are shown. AKT phosphorylation intensities were obtained by quantitative densitometric scanning of autoradiographc signals obtained with the phospho-specific AKT antibody and normalized with the signals obtained by the phospho-nonspecific AKT antibody. The relative band intensities are shown with the band densities from the cells treated with vehicle (ethanol, 0 μM of TAM) that were arbitrarily set as 1. The columns represent the means of three experiments; bars, SE.

4A). Like MCF7/ER36 cells, H3396 cells were more resistant to the growth inhibitory effects of TAM compared to the control MCF7 cells (Figure 4B).

Tamoxifen induces AKT activation in cells expressing ER-a36

Previously, we found that TAM elicited agonist activities such as activation of the MAPK/ERK and the PI3K/AKT pathways in ER-α36 expressing endometrial cells (15, 16). We sought to determine whether ER-α36 mediates agonist activity of TAM in cells with high concentrations of ER-α36. We first treated MCF7 cells with different concentrations of TAM and the AKT phosphorylation was measured with Western blot analysis. In control MCF7 cells transfected with the empty expression vector (MCF7/Vector), we found that at lower concentrations from 1 to 3 μM, TAM induced the AKT phosphorylation while at 4–5 μM failed to do so (Figure 5B). However, TAM at different concentrations failed to induce AKT activation in MCF7 cells with ER-α36 knocked-down (Figure 5 A & B), indicating ER-α36 mediates agonist activity of TAM. However, in MCF7/TAM, H3396 and MCF7/ER36 cells, TAM potently induced the AKT phosphorylation even at 4–5 μM (Figure 5C, D, E). In MCF7/TAM and H3396 cells, TAM induced AKT phosphorylation at 0.4 μM (Figure 5D, E). To further confirm the role of ER-α36 in the agonist activity of TAM, we also used MCF/TAM and H3396 cells with knocked-down concentrations of ER-α36 protein, and found that TAM failed to induce AKT phosphorylation in these cells (Figure 5D, E). Taken together, these results demonstrated that tamoxifen acts as an agonist to induce AKT phosphorylation in cells expressing ER-α36,

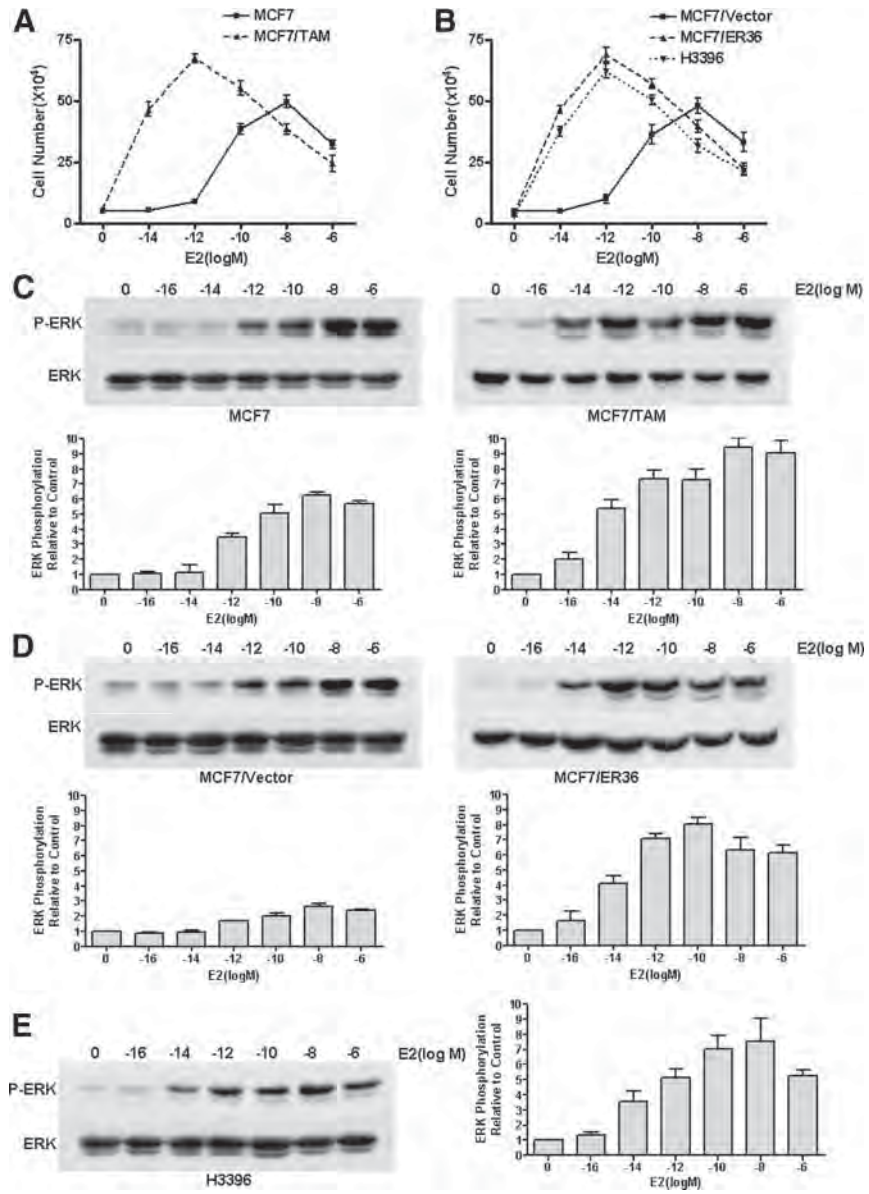

Figure 6. ER-α36 is involved in estrogen hypersensitivity. A. ER-positive breast cancer MCF7 cells and MCF7/TAM cells were treated with indicated concentrations of 17β-estradiol (E2) for seven days and cell number was counted. Each point represents the means of three experiments; bars, SE. B. MCF7 cells transfected with the empty expression vector (MCF7/Vector) and ER-α36 expression vector (MCF7/ER36) as well as H3396 cells were treated with indicated concentrations of E2 for seven days and cell number was determined. Each point represents the means of three experiments; bars, SE. C, D, E. Estrogen induces ERK activation in different cell lines. Western blot analysis of the lysates from different cells treated with indicated concentrations of E2 for 30 min using the phospho-specific or nonspecific ERK1/2 antibodies. All experiments were done at least three times and representative results are shown. ERK phosphorylation levels were obtained by quantitative densitometric scanning of autoradiographc signals obtained with the phospho-specific ERK antibody and normalized with the signals obtained by the phospho-nonspecific ERK antibody. The relative band intensities are shown with the band densities from the cells treated with vehicle (ethanol, 0 M of E2) that were arbitrarily set as 1. The columns represent the means of three experiments; bars, SE.

which provides an explanation to the involvement of ER-α36 in TAM resistance.

ER-a36 expressing breast cancer cells exhibit estrogen hypersensitivity

Previously, it was reported that cells deprived of estrogen for a long-term exhibited hypersensitivity to estrogens (8). We decided to examine whether ER-α36 is involved in development of estrogen hypersensitivity. MCF7/TAM cells were treated with different concentrations of 17β-estradiol (E2) for seven days. We found that E2 stimulated stronger proliferation in these cells compared to the parental MCF7 cells (Figure 6A). In addition, MCF7/TAM cells exhibited hypersensitivity to E2; at pM range, E2 stimulated proliferation of MCF/TAM cells while E2 stimulated proliferation of the parental MCF7 cells at nM range (Figure 6A). We also found that MCF7/ER36 cells that express recombinant ER-α36 and H3396 cells with high concentrations of endogenous ER-α36 protein also exhibited estrogen hypersensitivity (Figure 6B), suggesting that ER-α36 is involved in estrogen hypersensitivity.

We then examined E2-induced phosphorylation of the MAPK/ERK1/2, a typical nongenomic estrogen-signaling event, in different cell lines. Cells were treated with E2 at different concentrations for 30 min, and Western blot analysis with a phospho-specific ERK1/2 antibody was performed. Figure 6C shows that E2 elicited ERK phosphorylation in MCF/TAM cells in a dos-dependent manner starting at a very low concentration, 1×10^{-14} M/L, while in the parental MCF7 cells and MCF/Vector cells, ERK activation requires E2 at 1×10^{-12} M/L (Figure 6C, D). A similar hypersensitivity was also observed in MCF7/ER36 and H3396 cells (Figure 6D & E); E2 induced ERK phosphorylation at 1×10^{-14} M/L. Our data thus suggested that increased concentration of ER-α36 protein is one of the mechanisms underlying estrogen hypersensitivity.

Discussion

Tamoxifen therapy is the most effective treatment for advanced ER-positive breast cancer, but its effectiveness is limited by high rate of de novo resistance and resistance acquired during treatment. Many studies were conducted to understand the molecular pathways responsible for the de novo and acquired tamoxifen resistance, and have revealed that multiple signaling molecules and pathways are involved in tamoxifen resistance. All of these pathways often bypass the requirement of estrogen signaling pathway for growth of ER-positive breast cancer cells. Previously, we reported that the breast cancer patients with tumors expressing high concentrations of endogenous ER-α36 less benefited from tamoxifen therapy than those with low concentrations of ER-α36 (16), suggesting elevated concentration of ER-α36 protein may be a novel mechanism underlying both de novo and acquired tamoxifen resistance.

Here, we showed that tamoxifen treatment induced ER-α36 expression and tamoxifen resistant MCF7/TAM cells selected with long-term cultivation in the presence of tamoxifen expressed elevated concentration of ER-α36 protein. We also showed that MCF7 cells with forced ER-α36 expression and H3396 cells that express high concentration of endogenous ER-α36 protein were relatively more resistant to tamoxifen compared to MCF7 cells. Downregulation of ER-α36 expression, however, was able to restore tamoxifen sensitivity in MCF/TAM and H3396 cells, indicating that increased ER-α36 concentration is one of the molecular mechanisms by which ER-positive breast cancer develops tamoxifen resistance.

Previously, we found that antiestrogens TAM and ICI 182, 780 failed to block ER-α36-mediated nongenomic estrogen signaling (14). Here we showed that TAM exhibited a biphasic activation of the AKT kinase in tamoxifen-sensitive MCF7 cells; increasing AKT phosphorylation at low concentrations and failed to do so at higher concentrations. However, in cells with high concentrations of ER-α36 protein, TAM still activates the AKT kinase at higher concentrations, consistent with our recent report that ER-α36 mediates agonist activities of both TAM and ICI 182, 780 (18). Recently, loss of p21 (CDKN1A), a cyclin-dependent kinase inhibitor was found to be associated with the agonist activity of tamoxifen (20). Likewise, inhibition of p27 (CDKN1B), another cyclin-dependent kinase inhibitor, by Src has been associated with a tamoxifen-resistance phenotype (21). Both p21 and p27 are phosphorylated by the AKT kinase and this phosphorylation banishes both p21 and p27 from the cell nucleus and keeps them in the cytoplasm (22, 23). Thus, loss of expression and function, and relocalization of either of two G1-checkpoint CDK inhibitors after AKT phosphorylation can lead to TAM resistance. We found that in TAM sensitive MCF7 cells, TAM downregulated p27 phosphorylation and increased concentration of p27 protein whereas TAM upregulated p27 phosphorylation and decreased concentration of p27 protein (Zhang et al. unpublished data). Our results suggested that ER-α36 mediated agonist activity of TAM such as activation of the PI3K/AKT signaling is important for ER-α36 function in TAM resistance.

Previously, another acquired TAM resistance phenotype has been described in a human breast cancer xenograft model that exhibits a switch from a TAM-inhibitory phenotype to a TAM-stimulated one. Some breast cancers may be initially inhibited by TAM, and later become dependent on TAM for proliferation (24–26). These xenogafts also retain the ability to be stimulated by estrogens (24–26). In the current study, we found that 1 μM of TAM stimulated proliferation of MCF7/TAM cells while downregulation of ER-α36 expression in these cells diminished TAM-stimulation. In addition, these TAM resistant cells retained estrogen responsiveness, and even showed estrogen hypersensitivity. Our results thus suggested that elevated ER-α36 concentration is involved in this type of TAM resistance. It also worth noting that the TAM at 1 μM failed to stimulate proliferation of MCF7/36 cells that express recombinant ER-α36 and H3396 that express endogenous ER-α36 (Figure 4B). The exact mechanism for this is not known. We observed that TAM-resistant MCF7/TAM cells also gained expression of the growth factor receptors EGFR and HER2 while MCF7/ER36 cells mainly increased the concentration of EGFR protein and H3396 cells only express modest concentration of EGFR. Thus, it is possible that increased expression or signaling of the HER2 receptor in MCF7/TAM cells contributes to the TAM-stimulated proliferation in MCF7/TAM cells. Intriguingly, the expression of both EGFR and HER2 were downregulated in MCF7/TAM/Si36 cells, consistent with our recent reports there are positive regulatory loops between ER-α36 and the EGFR/HER2 expression; ER-α36 stabilizes EGFR protein and activates HER2 promoter activity while the signaling of EGFR/HER2 induces ER-α36 expression (19, 31).

Previously, it has been reported that physiological concentrations of E2 exhibit antitumor activity in a TAM-stimulatory MCF7 cell model that was generated by serial transplantation of TAM resistant tumors in the continuous presence of TAM (27). Based on the laboratory studies, it was recently proposed that physiological concentration of estrogen could be used as a therapeutic approach for these TAM resistant patients (28, 29). However, the molecular mechanisms underlying this paradox-

ical phenomenon have not been well elucidated. It is known that estrogen stimulates growth of ER-positive breast cancer cells in a biphasic growth curve; stimulating cell proliferation at low concentrations while failing to stimulate or even inhibiting cell growth at higher concentrations. Our results presented here that elevated ER-α36 concentration rendered cells hypersensitive to E2; shifting the biphasic growth curve to the left. Thus, in cells expressing high concentrations of ER-α36 protein, physiological concentrations of E2 may fail to stimulate proliferation or even inhibit proliferation. Our data thus provided a molecular explanation to the paradoxical phenomenon that some TAM resistant tumors are simulated by TAM but inhibited by estrogen.

Previously, it was reported that long-term estrogen deprivation with hormonal therapy resulted in "adaptive" changes of breast cancer cells; making these cells hypersensitive to estrogen (8, 11). Recently, we reported that ER-α36 concentration is significantly increased in normal osteoblasts cells from menopausal women (30), suggesting that ER-α36 expression is elevated in response to low concentration of estrogen in menopausal women. Our current data showed that E2 induced ERK phosphorylation and stimulated proliferation at pM range in cells with high concentration of ER-α36 protein while at nM range in cells with low concentration of ER-α36. Thus, our results indicated that gained ER-α36 expression is one of the "adaptive" changes in breast cancer cells after a long-term estrogen deprivation resulted from antiestrogen treatment.

In summary, here we provided evidence to demonstrate that ER-α36 is a novel and important player in normal and abnormal estrogen signaling, and ER-α36 is involved in many physiological and pathological processes regulated by estrogen signaling. Our findings that elevated ER-α36 concentration is one of the mechanisms by which ER-positive breast cancer cells escape the antiestrogen therapy provided a rational to develop novel therapeutic approaches for antiestrogen resistant patients by targeting ER-α36.

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Author Disclosure Summary: X.T.Z and Z.Y.W. have nothing to declare.

This work was supported by Department of Defense grant DAMD 11–1-0497 and Nebraska Tobacco Settlement Biomedical Research Program Awards (LB-595) to Z.Y. Wang.

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European Journal of Pharmacology 714 (2013) 56–64

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Endocrine pharmacology

A novel anticancer agent Broussoflavonol B downregulates estrogen receptor (ER)-α36 expression and inhibits growth of ER-negative breast cancer MDA-MB-231 cells

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ARTICLE INFO

Article history:
Received 10 April 2013
Received in revised form
24 May 2013
Accepted 30 May 2013
Available online 11 June 2013

Keywords:
ER-negative breast cancer
Breast cancer stem cells
Broussoflavonol B
ER-α36
EGFR

ABSTRACT

Estrogen receptor (ER)-negative breast cancers are aggressive and unresponsive to antiestrogens, and current therapeutic modalities for ER-negative breast cancer patients are usually associated with strong toxicity and side effects. Less toxic and more effective targeted therapies are urgently needed to treat this type of breast cancer. Here, we report that Broussoflavonol B, a chemical purified from the bark of the Paper Mulberry tree (*Broussonetia papyrifera*) exhibited potent growth inhibitory activity in ER-negative breast cancer MDA-MB-231 cells at sub-micromolar concentrations. Broussoflavonol B induced cell cycle arrest at both the G_0/G_1 and G_2/M phases accompanied by a downregulation of c-Myc protein, a upregulation of the cell cycle inhibitory proteins $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$ and a down-regulation of the expression levels of the G_2/M regulatory proteins such as cyclin B1, cdc2 and cdc25C. Broussoflavonol B also induced apoptotic cell death characterized by accumulation of the annexin V- and propidium iodide-positive cells, and cleavage of caspases 8, 9 and 3. In addition, Broussoflavonol B treatment also decreased the steady state levels of the epidermal growth factor receptor (EGFR) and ER-α36, a variant of estrogen receptor-α, and restricted growth of the stem-like cells in ER-negative breast cancer MDA-MB-231 cells. Our results thus indicate that Broussoflavonol B is a potent growth inhibitor for ER-negative breast cancer cells and provide a rational for preclinical and clinical evaluation of Broussoflavonol B for ER-negative breast cancer therapy.

1. Introduction

Depending on the existence of one of the estrogen receptors, ER-α, human breast cancers are divided into ER-positive or ER-negative. Approximately 70% of breast cancer patients are positive for ER-α and these patients are suitable for hormonal therapy that blocks estrogen stimulation of breast cancer cells. However, ER-negative breast cancer that accounts for about one third of breast cancers diagnosed is often more malignant and aggressive than ER-positive breast cancer. In addition, ER-negative breast cancer patients respond poorly to antiestrogen therapy, and current therapeutic modalities for ER-negative breast cancer patients are usually associated with strong toxicity and side effects. Less toxic and more effective targeted therapeutic approaches are urgently needed to treat this type of breast cancer.

Previously, we identified and cloned a 36 kDa variant of ER-α, ER-α36, that is mainly expressed outside of the cell nucleus and mediates non-genomic estrogen signaling (Wang et al., 2005, 2006). ER-α36 lacks both transcription activation function domains AF-1 and AF-2 of the full-length 66 kDa ER-α (ER-α66), consistent with the fact that ER-α36 has no intrinsic transcriptional activity (Wang et al., 2006). ER-α36 is generated from a promoter located in the first intron of the ER-α66 gene (Zou et al., 2009), indicating that ER-α36 expression is regulated differently from ER-α66, consistent with the findings that ER-α36 is expressed in specimens from ER-negative breast cancer patients and established ER-negative breast cancer cells that lack ER-α66 expression (Pelekanou et al., 2012; Shi et al., 2009; Vranic et al., 2011; Wang et al., 2006). ER-α36 is critical for malignant growth of ER-negative breast cancer cells (Zhang et al., 2011); ER-negative breast cancer MDA-MB-231 and -436 cells with knocked-down concentrations of ER-α36 protein failed to form xenograft tumors in nude mice. Thus, ER-α36-mediated signaling plays an important role in development and progression of ER-negative breast cancer, and ER-α36 may be used as a target to develop novel and more

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http://dx.doi.org/10.1016/j.ejphar.2013.05.047

effective therapeutic agents for treatment of ER-negative breast cancer.

Broussonetia papyrifera (Moraceae), also known as paper mulberry, grows naturally in Asia and Pacific countries. Its dried fruits have been used for the treatment of ophthalmic disorders and impotency (Lee et al., 2001). The leaves, twig roots and barks of this plant are widely used to treat gynecological bleeding, dropsy, dysentery diseases as a folk medicine in China (Feng et al., 2008). Various types of flavonoids are the major constituents of this plant and some of which exhibited strong tyrosinase inhibitory (Zheng et al., 2008), aromatase inhibitorory (Lee et al., 2001), antifungal (Takasugi et al., 1980, 1984), secretory phospholipase A-2 inhibitory (Kwak et al., 2003), PTP1B enzyme inhibitory (Chen et al., 2002; Nguyen et al., 2012), antimicrobial, cytotoxic (Sohn et al., 2004), antiplatelet (Lin et al., 1996), antioxidant and inducible nitric oxide synthase suppressing activities (Cheng et al., 2001). However, the effects and the underlying mechanisms of the flavonoids from *B. papyrifera* in human cancer have never been studied. Recently, we purified and identified two prenylflavone derivatives from *B. papyrifera* (Guo et al., 2013); one is a known compound Broussonol D (Zhang et al., 2011) that has been isolated from Broussonetia kazinoki and another is Broussoflavonol B (Matsumoto et al., 1985). We also found that both compounds were able to inhibit growth of ER-positive breast cancer MCF7 cells presumably through down-regulation of ER-α36 expression (Guo et al., 2013). However, Broussoflavonol B was more potent than Broussonol D in downregulation of ER-α36 expression (Guo et al., 2013). Since ER-α36 is critical for malignant growth of ER-negative breast cancer cells (Zhang et al., 2011), we decided to study the effects and underlying mechanisms of Broussoflavonol B (5,7,3′,4′-Tetrahydroxy-3-methoxy-6,8-diprenylflavone) purified from the bark of *B. papyrifera* in growth of ER-negative breast cancer MDA-MB-231 cell.

In the present study, we demonstrated that Broussoflavonol B exhibited potent growth inhibitory activity in ER-negative breast cancer MDA-MB-231 cells at sub-micromolar (μM) concentrations. Broussoflavonol B treatment decreased the steady state levels of ER-α36 and EGFR proteins, and induced cell cycle arrest and cell apoptosis.

2. Materials and methods

2.1. Chemicals and reagents

Broussoflavonol B (99.5% purity) was obtained from Shenogen Parma Group, Ltd (Beijing, China). Anti-p16^{INK4a} (N-20), p19^{INK4D} (M-167), p21$^{WAF1/CIP1}$ (F-5), β-actin (I-19), c-Myc (9E10), caspase 3 (S-19), caspase 8 (H-134), caspase 9 (H-170), Cdc 25c (H-150), cyclin B1 (GNS1) and Cdc2 p34 (POH-1), cytokeratin 18 (DC-10), CD10 (H-321) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-EGFR antibody (1F4) and anti-vimentin (D21H3) antibody were from Cell Signaling Technology (Danvers, MA, USA). Polyclonal anti-ER-α36 antibody was generated and characterized as described before (Wang et al., 2006).

2.2. Cell culture

MDA-MB-231 cells were purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution from Invitrogen Life Technologies Corporation (Carlsbad, CA, USA). Before experiments, cells were maintained in phenol red-free media with 2.5% charcoal-stripped FBS (Thermo Scientific Hyclone, Logan, UT, USA).

2.3. Cell growth and differentiation assays

Cells in the phenol red-free medium were seeded onto 35 mm dishes at 5×10^4 cells/dish. After 24 h, the indicated concentrations of vehicle dimethyl sulfoxide (DMSO), Broussoflavonol B or tamoxifen were added and incubated for seven days. Cells were trypsinized and counted using the ADAM automatic cell counter (Digital Bio, Korea). Three dishes were used for each concentration point and experiments were repeated at least three times.

To assess the effects of Broussoflavonol B on epidermal growth factor (EGF)-stimulated cell growth, cells (1×10^4/dish) in 60 mm dishes were maintained in phenol red-free medium with 2.5% charcoal-stripped FBS (Thermo Scientific Hyclone, Logan, UT, USA) for 48 h. EGF (10 ng/ml) alone, together with Broussoflavonol B or Broussoflavonol B alone were added to cells and incubated for 72 h, and the cell numbers were determined using the ADAM automatic cell counter (Digital Bio., Korea). Three dishes were used for each treatment and experiments were repeated more than three times.

For cancer stem-like cell growth, MDA-MB-231 cells were seeded onto Corning Ultra-Low Attachment 6-well plate (Corning Incorporated, CA, USA) at 10,000 cells/ml and cultured in phenol red-free DMEM/F12 medium (Invitrogen) supplemented with 1X B27 (Invitrogen), 20 ng/ml epidermal growth factor (Sigma-Aldrich, St. Louis, MO, USA) and 20 ng/ml basic fibroblast growth factor (ProSpec, NJ, USA), 0.5 μg/ml hydrocortisone (Sigma). The different concentrations of Broussoflavonol B or tamoxifen were

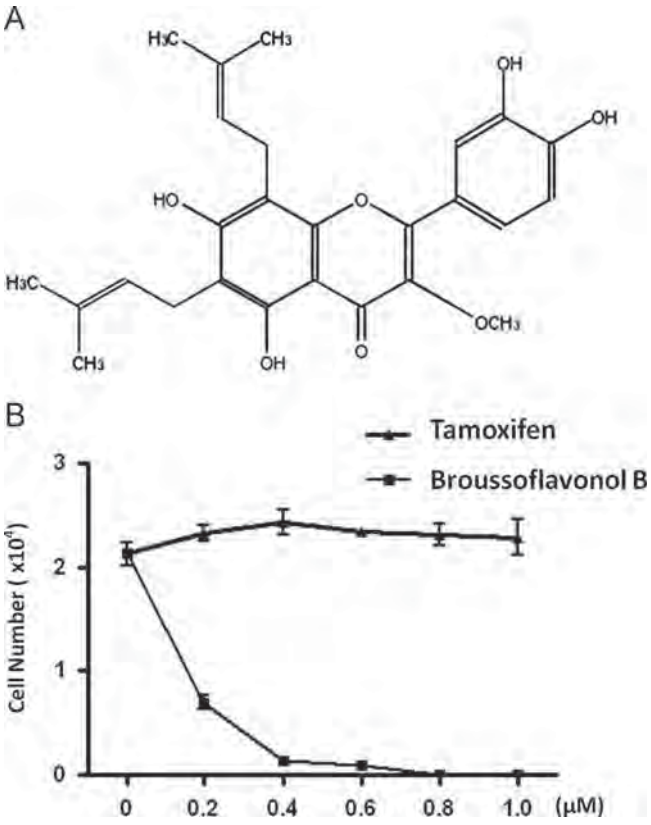

Fig. 1. Broussoflavonol B inhibits growth of ER-negative breast cancer MDA-MB-231 cells. (A) The chemical structure of Broussoflavonol B (5,7,3′,4′-Tetrahydroxy-3-methoxy-6,8-diprenylflavone). (B) Effects of Broussoflavonol B or tamoxifen on the growth of MDA-MB-231 cells. Cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with vehicle, DMSO (0) and 0.2, 0.4, 0.6, 0.8 and 1.0 μM of Broussoflavonol B or tamoxifen for seven days before cells were trypsinized and counted. Three dishes were used for each concentration in the experiments and all experiments were repeated three times. Each point represents mean ± S.D. of three independent experiments.

added and incubated for seven days. Cells were collected, washed with PBS, and incubated with Trypsin-EDTA (0.05%/0.5 mM) for 2 min at 37 °C, and cells were counted using the ADAM automatic cell counter (Digital Bio, Korea).

For the differentiation assay of cancer stem-like cells, MDA-MB-231 cells were seeded onto Corning Ultra-Low Attachment 10 cm dishes (Corning Incorporated) at 10,000 cells/ml and cultured in the stem cell medium for seven days. Indicated concentrations of Broussoflavonol B were added and incubated for another three days. Cells were collected, washed with phosphate buffered saline (PBS) and cytospined onto slides. Cytospined slides were stained with indirect immunofluorescent staining using anti-CD10, vimentin or CK18 antibodies. Five hundred cells were assessed for vimentin or CK18 positivity under the fluorescent microscope (Nikon, Eclipss E600), and the percentage of cells positive for these markers were calculated.

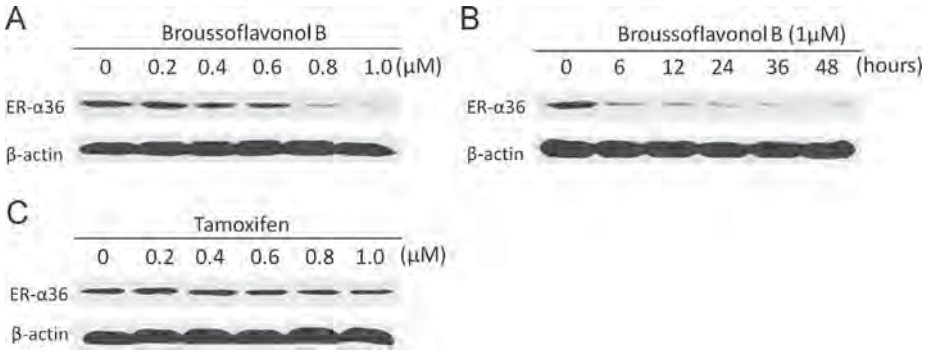

Fig. 2. Broussoflavonol B treatment downregulates ER-α36 expression. ((A) and (B)) MDA-MB-231 cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with DMSO (0), and indicated concentrations of Broussoflavonol B for 12 h or 1 μM of Broussoflavonol B for indicated time periods. Cell lysates were subjected to Western blot analysis with an antibody for ER-α36. (C) Cells were treated with DMSO (0) and indicated concentrations of tamoxifen. Cell lysates were subjected to Western blot analysis with the antibody for ER-α36. All membranes were stripped and re-probed with a β-actin antibody to ensure equal loading.

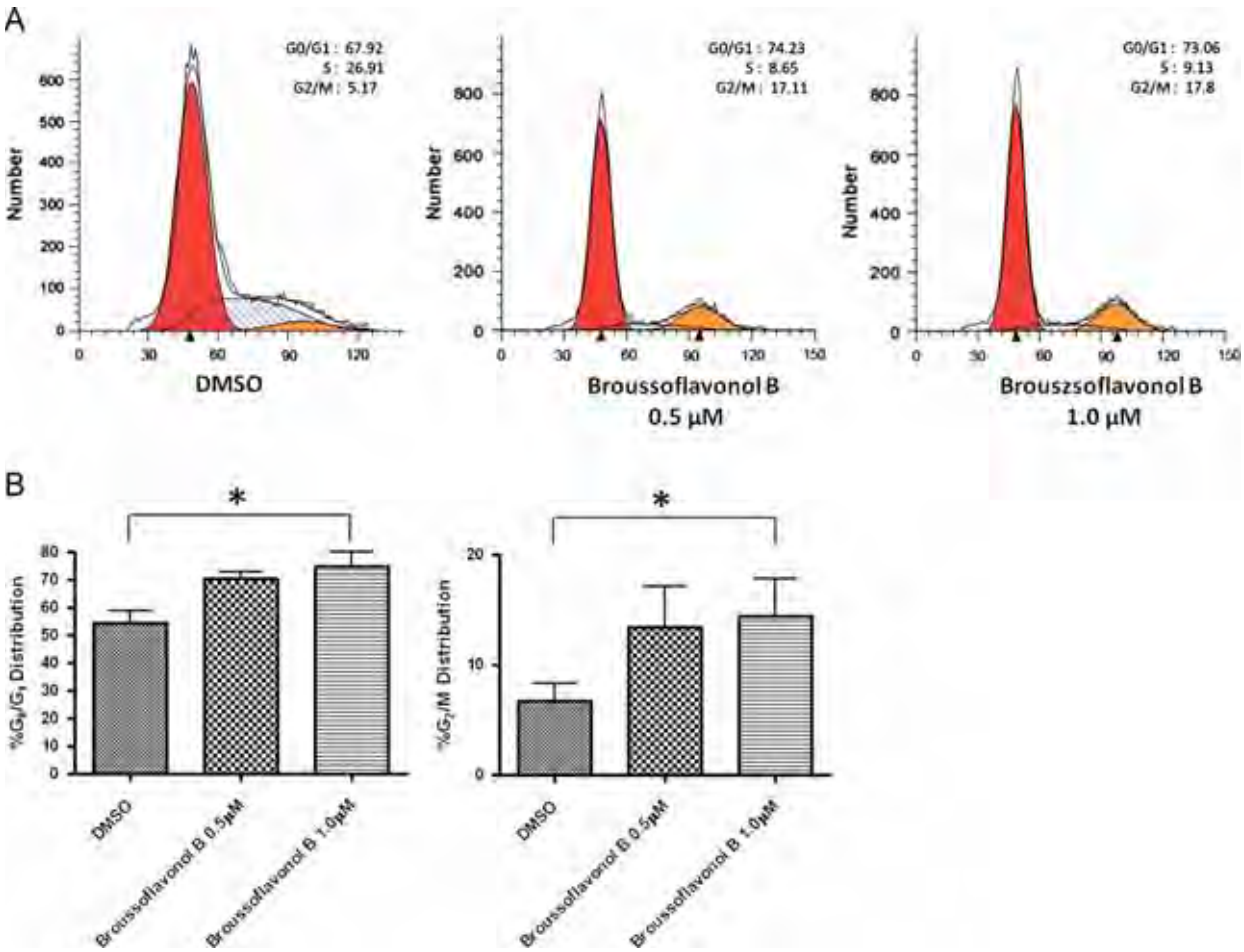

Fig. 3. Broussoflavonol B induces G_0/G_1 and G_2/M arrest of the cell cycle in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with DMSO (vehicle) or the indicated concentrations of Broussoflavonol B for 72 h. Cells were assayed with PI staining and flow cytometric analysis. All experiments were repeated three times and data from a representative experiment is shown. (B) Percentage of cells in the G_0/G_1 and G_2/M phases of the cell cycle in MDA-MB-231 cells treated with or without Broussoflavonol B. Each column represents mean ± S.D. of three independent experiments. * $P < 0.05$.

2.4. Western blot assay

Cells were washed with cold PBS twice and lysed with the RIPA buffer containing 1% proteinase inhibitor and 1% phosphatase inhibitor cocktail solution (Sigma, St. Louis, MO, USA). The cell lysates were boiled for 5 min in sodium dodecyl sulfate (SDS) gel-loading buffer and separated on 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with appropriate primary antibodies and visualized with the corresponding secondary antibodies and the ECL kit (Thermo Scientific, Rockford, IL, USA).

2.5. Cell cycle and cell death analysis

Cells at ~70% confluence were harvested and 1 ml of cold 70% ethanol was slowly added to the cell pellet while vortexing. Ethanol-fixed cells were treated with 100 μg/ml RNaseA and 50 μg/ml propidium iodide (PI) in PBS at room temperature for 30 min. Flow cytometry analysis of cell cycle distribution was performed using a FACSCalibur flow cytometer (BD-Biosciences).

Cell death was detected using the annexin V-FITC apoptosis kit (Invitrogen) according to the manufacturer's instruction. Data acquisition was performed with the CellQuest software and analyzed with the ModFit software.

2.6. Statistical analysis

Data were summarized as the means ± standard deviation (S.D.) using GraphPad InStat software program. Statistical analysis was performed using paired-samples *t*-test, or ANOVA followed by the Student–Newman–Keuls testing and the significance was accepted for *P* values less than 0.05.

3. Results

3.1. Broussoflavonol B exhibits growth inhibitory activity in ER-negative breast cancer MDA-MB-231 cells

Previously, we reported that ER-α36 plays an critical role in malignant growth of ER-negative breast cancer MDA-MB-231 cells (Zhang et al., 2011), suggesting the downregulation of ER-α36 expression may provide a novel approach to inhibit growth of ER-negative breast cancer cells. Recently, we found that a flavonoid, Broussoflavonol B (5, 7, 3′, 4′-Tetrahydroxy-3-methoxy-6,8-diprenylflavone) (Fig. 1A) purified from the bark of *B. papyrifera* was able to downregulate ER-α36 expression and inhibit proliferation in ER-positive breast cancer MCF7 cells (Guo et al., 2013).

To examine the effects of this chemical on growth of ER-negative breast cancer cells, we used ER-negative breast cancer MDA-MB-231 cells as a model to perform cell growth inhibition assay. Cells were incubated with increasing concentrations of Broussoflavonol B or the classical anti-estrogen tamoxifen for seven days, and the cell numbers were then counted. Broussoflavonol B potently inhibited growth of MDA-MB-231 cells at sub-μM concentrations while the classical anti-estrogen tamoxifen had no effect (Fig. 1B). Our result thus suggested that Broussoflavonol B exhibited growth inhibitory activity in ER-negative breast cancer cells.

3.2. Broussoflavonol B downregulates ER-α36 expression in MDA-MB-231 cells

To probe the molecular mechanisms by which Broussoflavonol B inhibited growth of MDA-MB-231 cells, we assessed the effects of Broussoflavonol B on expression of ER-α36, a protein important for malignant growth of MDA-MB-231 cells (Zhang et al., 2011). Western blot analysis indicated that Broussoflavonol B treatment downregulated ER-α36 expression in a dose- and time-dependent manner (Fig. 2A and B) whereas the classic anti-estrogen tamoxifen was without any effect on ER-α36 expression (Fig. 2C). Thus, our data suggested that ER-α36 downregulation is a mechanism underlying Broussoflavonol B growth inhibitory activity in these cells.

3.3. Broussoflavonol B induces both the G_0/G_1 and G_2/M phase arrest in MDA-MB-231 cells

To further examine the mechanisms underlying Broussoflavonol B growth inhibitory activity, we also studied its effect on the cell cycle progression. Cell populations in the G_0/G_1, S and G_2/M phases of the cell cycle were determined with propidium iodide (PI) staining followed by flow cytometry. Broussoflavonol B treatment increased the population of MDA-MB-231 cells in both the G_0/G_1 and G_2/M phases accompanied with a dramatically reduced population of the S phase; for vehicle (DMSO), 0.5 and 1 μM Broussoflavonol B, respectively (Fig. 3A and B).

We also examined the effects of Broussoflavonol B on the expression of the proteins involved in regulation of the G_1/S transition of the cell cycle including c-Myc, $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$. Western blot analysis showed that Broussoflavonol B treatment down-regulated the expression levels of the growth promoting protein c-Myc in a dose-dependent manner (Fig. 4A) while induced expression levels of the cell cycle inhibitory proteins $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$ (Fig. 4A).

We then examined the effect of Broussoflavonol B on the expression of the proteins critical for the G_2/M transition including cyclin B1, cdc2 and cdc25C. Western blot analysis showed that

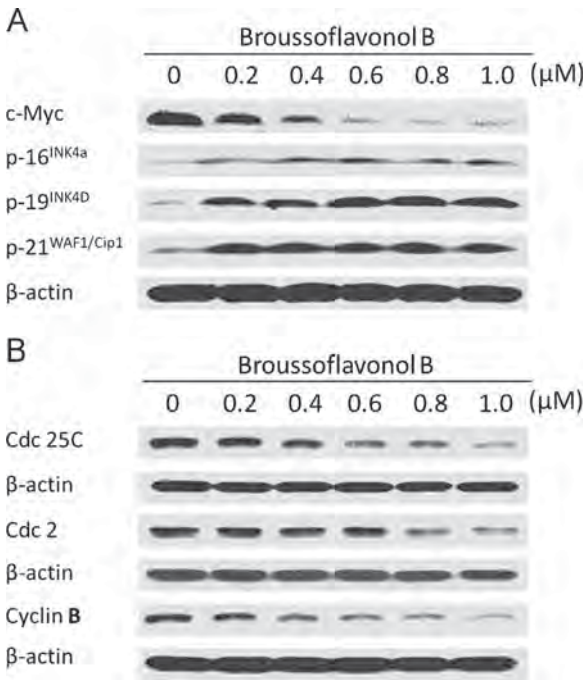

Fig. 4. Broussoflavonol B regulates expression levels of the cell cycle regulators in MDA-MB-231 cells. (A) MDA-MB-231 cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with DMSO (0), and indicated concentrations of Broussoflavonol B for 12 h. Cell lysates were subjected to Western blot analysis with antibodies for c-Myc, $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$. (B) MDA-MB-231 **c**ells were treated with DMSO (0), and indicated concentrations of Broussoflavonol B for 12 h. Cell lysates were subjected to Western blot analysis with antibodies for cyclin B1, cdc2 and cdc25C. All membranes were stripped and re-probed with a β-actin antibody to ensure equal loading.

Broussoflavonol B treatment down-regulated the expression levels of cyclin B1, cdc25C and cdc2 in a dose-dependent manner (Fig. 4B). Altogether, these results demonstrated that Broussoflavonol B treatment arrested ER-negative breast cancer MDA-MB-231 cells at both the G_0/G_1 and G_2/M phases of the cell cycle.

3.4. Broussoflavonol B activates caspase-mediated cell apoptosis in MDA-MB-231 cells

During our experiments, we also noticed that there were floating cells in the MDA-MB-231 cells treated with Broussoflavonol B. We decided to determine whether Broussoflavonol B also induces cell apoptosis. MDA-MB-231 cells were treated with different concentrations of Broussoflavonol B for 48 h, and the annexin V-FITC and propidium iodide (PI) fluorescence assays were performed to examine the early stage apoptotic cells (annexin-positive/PI-negative), the late stage apoptotic cells (annexin-positive/PI-positive), and necrotic cells (annexin-positive/PI-positive). In MDA-MB-231 cells treated with 0.5 and 1 μM of Broussoflavonol B, cells were induced to apoptotic and/or necrotic cell death as shown as increased cell populations in groups of annexin V-positive/ PI-negative, annexin V-positive/PI-positive and annex V-negative/PI-positive (Fig. 5A and B).

We also examined whether the caspase cascades were activated in Broussoflavonol B treated cells. Western blot analysis revealed that Broussoflavonol B treatment resulted in a dose-dependent activation of the initiator caspases 8, 9 and the executor caspase 3. As shown in Fig. 5C, the levels of remaining procaspases 8, 9 and 3 in Broussoflavonol B treated cells were dramatically decreased compare to the control cells treated with DMSO vehicle. Taken together, our results demonstrated that Broussoflavonol B induces apoptotic as well as necrotic cell death in ER-negative breast cancer MDA-MB-231 cells.

3.5. Broussoflavonol B downregulates EGFR expression and inhibits EGF-stimulated growth of MDA-MB-231 cells

Recently, we reported that ER-α36 positively regulates the stability of the epidermal growth factor receptor (EGFR) protein; knockdown of ER-α36 expression destabilized EGFR protein (Zhang et al., 2011). We decided to examine whether down-regulated ER-α36 expression by Broussoflavonol B also down-regulates EGFR expression. We thus examined EGFR expression in MDA-MB-231 cells treated with Broussoflavonol B, and found that Broussoflavonol B also decreased the steady state levels of EGFR protein (Fig. 6A). MDA-MB-231 cells represent a typical triple-negative breast cancer that lacks expression of estrogen receptor, progesterone receptor and HER2, and often relies on EGFR signaling for malignant growth. We then decided to examine whether Broussoflavonol B is able to inhibit EGF-stimulated cell growth in MDA-MB-231 cells. In serum-starved cells, addition of

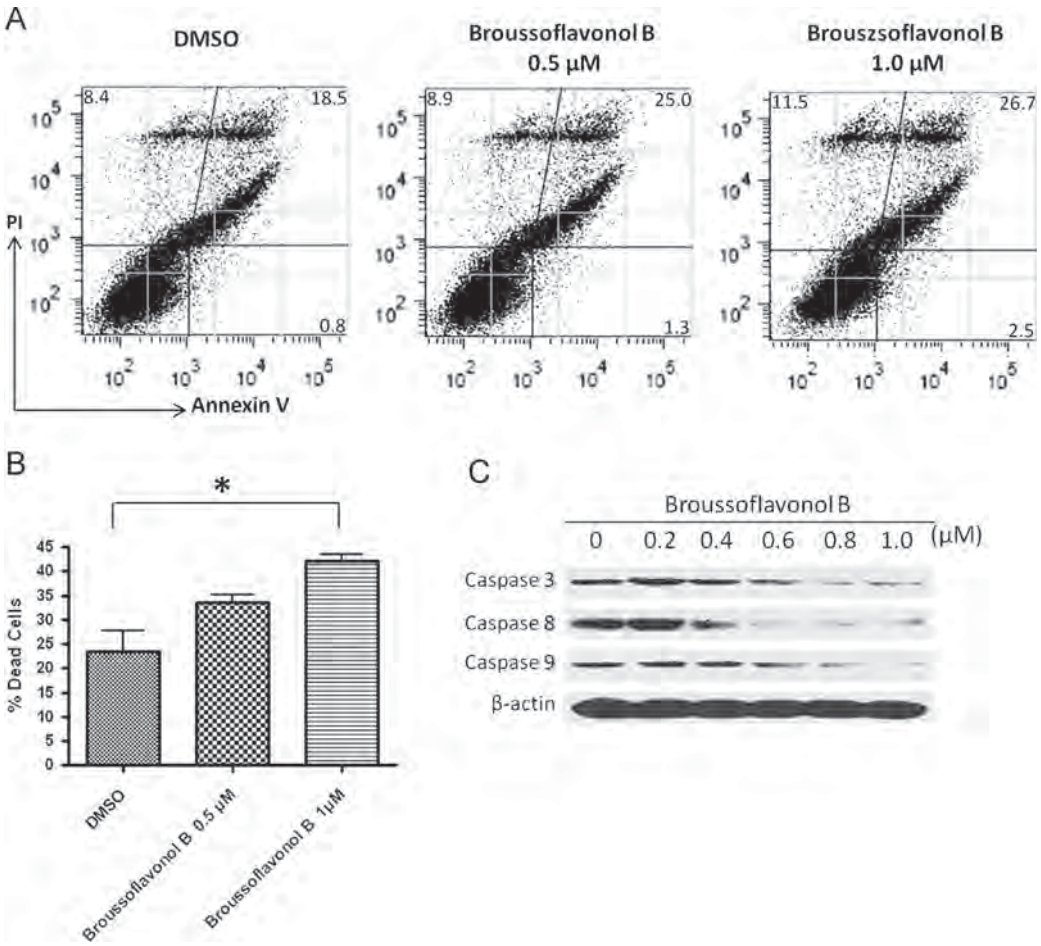

Fig. 5. Broussoflavonol B induces cell death in MDA-MB-231 cells. (A) MDA-MB-231 cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with DMSO (0) or 0.5 and 1 μM Broussoflavonol B for 48 h. Cells were collected and analyzed with flow cytometric analysis after annexin V and PI staining. The experiment was repeated three times and the results from a representative experiment were shown. (B) Percentage of dead cells in MDA-MB-231 cells treated with or without Broussoflavonol B. Each column represents mean ± S.D. of three independent experiments. * $P < 0.05$. (C) MDA-MB-231 cells were treated with DMSO (0), and indicated concentrations of Broussoflavonol B for 12 h. Cell lysates were subjected to Western blot analysis with antibodies for caspases 3, 8 and 9. All membranes were stripped and re-probed with a β-actin antibody to ensure equal loading.

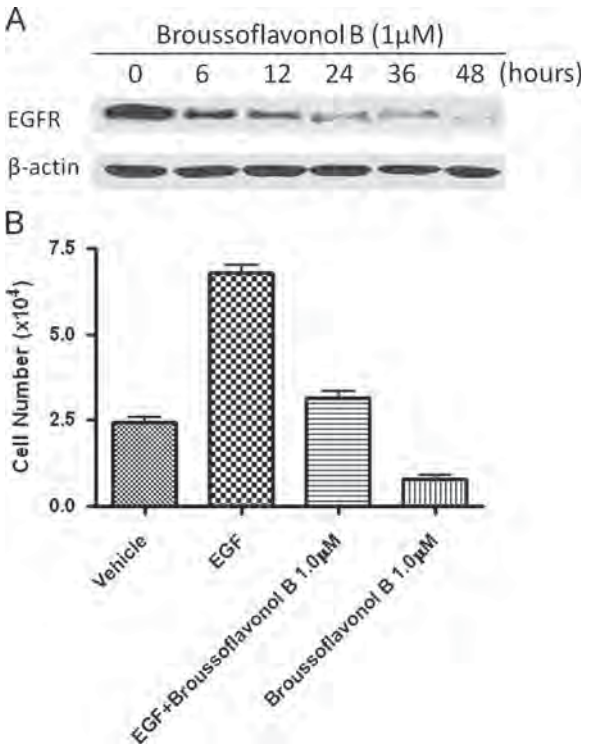

Fig. 6. Broussoflavonol B downregulates EGFR expression and attenuates mitogenic EGF signaling in MDA-MB-231 cells. (A) MDA-MB-231 **c**ells were treated with 1 μM of Broussoflavonol B for indicated time periods. Cell lysates were subjected to Western blot analysis with an antibody for EGFR. The membrane was stripped and re-probed with a β-actin antibody to ensure equal loading. (B) Cells maintained in phenol red-free medium with 2.5% charcoal-stripped FBS for 48 h. EGF (10 ng/ml) alone, together with 1 μM Broussoflavonol B or 1 μM Broussoflavonol B alone were added to cells and incubated for 72 h, and the cell numbers were determined. Three dishes were used for each treatment and experiments were repeated more than three times.

EGF (10 ng/ml) stimulated cell proliferation, which was inhibited by inclusion of Broussoflavonol B, indicating that Broussoflavonol B also attenuates mitogenic EGF signaling presumably via down-regulation of EGFR expression.

3.6. Broussoflavonol B inhibits the growth of breast cancer stem-like cells in MDA-MB-231 cells

Recently, we reported that ER-α36 is important in maintenance of the stem-like cells in ER-negative breast cancer SK-BR-3 cells (Kang et al., 2011). We decided to test the inhibitory effects of Broussoflavonol B on the stem-like cells in MDA-MB-231 cells. To this aim, we cultured MDA-MB-231 cells in a stem cell medium using ultralow-attachment dishes, which enriched the breast cancer stem-like cells. These stem-like cells were than treated with the increasing concentrations of tamoxifen or Broussoflavonol B for seven days. We found that Broussoflavonol B effectively inhibited the growth of these stem-like breast cancer cells while tamoxifen had less effect (Fig. 7A).

To examine whether Broussoflavonol B induces differentiation of breast cancer stem-like cells, we treated MDA-MB-231 cells cultured in the stem cell medium with indicated concentrations of Broussoflavonol B for three days. The cells were then examined for expression of differentiation markers including cytokeratin 18 (CK18) for luminal epithelial differentiation, CD10 for myoepithelial cell differentiation and vimentin for mecenchymal cell differentiation. We found that Broussoflavonol B treatment significantly increased the number of cells positive for CK18 while had no effect on the number of cells positive for vimentin (Fig. 7B). We did not observe CD10 staining in Broussoflavonol B treated cells (data not shown). Our results indicated that Broussoflavonol B is able to induce differentiation of ER-negative breast cancer stem-like cells mainly into the luminal epithelial lineage and differentiation induction may be one of the mechanisms by which Broussoflavonol B restricts growth of ER-negative breast cancer stem-like cells.

4. Discussion

In this study, we investigated the growth inhibitory potential of a flavonoid derivative Broussoflavonol B from the Paper Mulberry tree (*B. papyrifera*). *B. papyrifera* has been used for cancer, dyspepsia, and pregnancy (Johnson, 1998). In mainland China, the fruits of *B. papyrifera* have been employed for impotency and ophthalmic disorders (Matsuda et al., 1995). Crude extracts or purified compounds from *B. papyrifera* have exhibited various biological activities, such as anti-proliferation, antioxidative, aromatase inhibitory, cytotoxic, glycosidase inhibitory, and platelet aggregation inhibitory effects (Lee and Kinghorn, 2003).

Currently, seven broussoflavonols were purified from *B. papyrifera* and named as broussoflavonols A through G (Lee and Kinghorn 2003). Broussoflavonols E and F exhibited platelet aggregation inhibitory activity partially due to an inhibitory effect on cyclooxygenase (Lin et al., 1996). Both Broussoflavonols F and G potently inhibited Fe^{2+}-induced lipid oxidation in rat-brain homogenate and significantly inhibited the proliferation of rat vascular smooth muscle cells (Ko et al., 1997). Recently, we reported that Broussoflavonol B significantly inhibited growth of ER-positive breast cancer cells (Guo et al., 2013). Here, we demonstrated that Broussoflavonol B also potently inhibited growth of triple-negative and basal-like breast cancer MDA-MB-231 cells through down-regulation of ER-α36 and EGFR expression and induction of the G_0/G_1 and G_2/M arrest of the cell cycle as well as cell death. We also showed that Broussoflavonol B reduced the population of breast cancer stem-like cells.

The ER-α variant, ER-α36, is highly expressed in ~40% of ER-negative breast cancer (Shi et al., 2009) and its expression is significantly correlated with expression of members of the EGFR family such as EGFR and HER2 (Shi et al., 2009; Zhang et al., 2011). Recently, we reported the existence of a positive feedback loop between EGFR and ER-α36 expression in ER-negative breast cancer cells, which is critical for malignant growth of ER-negative breast cancer cells (Zhang et al., 2011). Here, we reported that Broussoflavonol B potently downregulated ER-α36 expression at sub-μM while antiestrogen tamoxifen was without any effects. In addition, Broussoflavonol B also downregulated the levels of EGFR protein since ER-α36 protein is important for stability of EGFR protein (Zhang et al., 2011), and attenuated the mitogenic EGF signaling that is critical for malignant growth of MDA-MB-231 cells. Thus, our results suggested that disruption of the positive regulatory loop between ER-α36 and EGFR through down-regulation of ER-α36 provides an effective approach to inhibit growth of ER-negative breast cancer cells.

Eukaryotic cell cycle progression involves sequential activation of Cdks, which are controlled by a complex of proteins, including the cyclins. Here, we found that Broussoflavonol B treatment also arrested MDA-MB-231 cells mainly at the G_2/M phase of the cell cycle, which was accompanied with down-regulation of the expression levels of the proteins pivotal for the G_2/M transition. We also found that Broussoflavonol B modestly arrested the cell cycle at the G_0/G_1 phase. Cell-cycle progression involves sequential activation of cyclins and cyclin-dependent kinases (CDKs). To prevent abnormal proliferation, cyclin-CDK complexes are negatively regulated by cell cycle inhibitors (Sherr and Roberts, 1999). Here, we found that in Broussoflavonol B treated MDA-MB-231

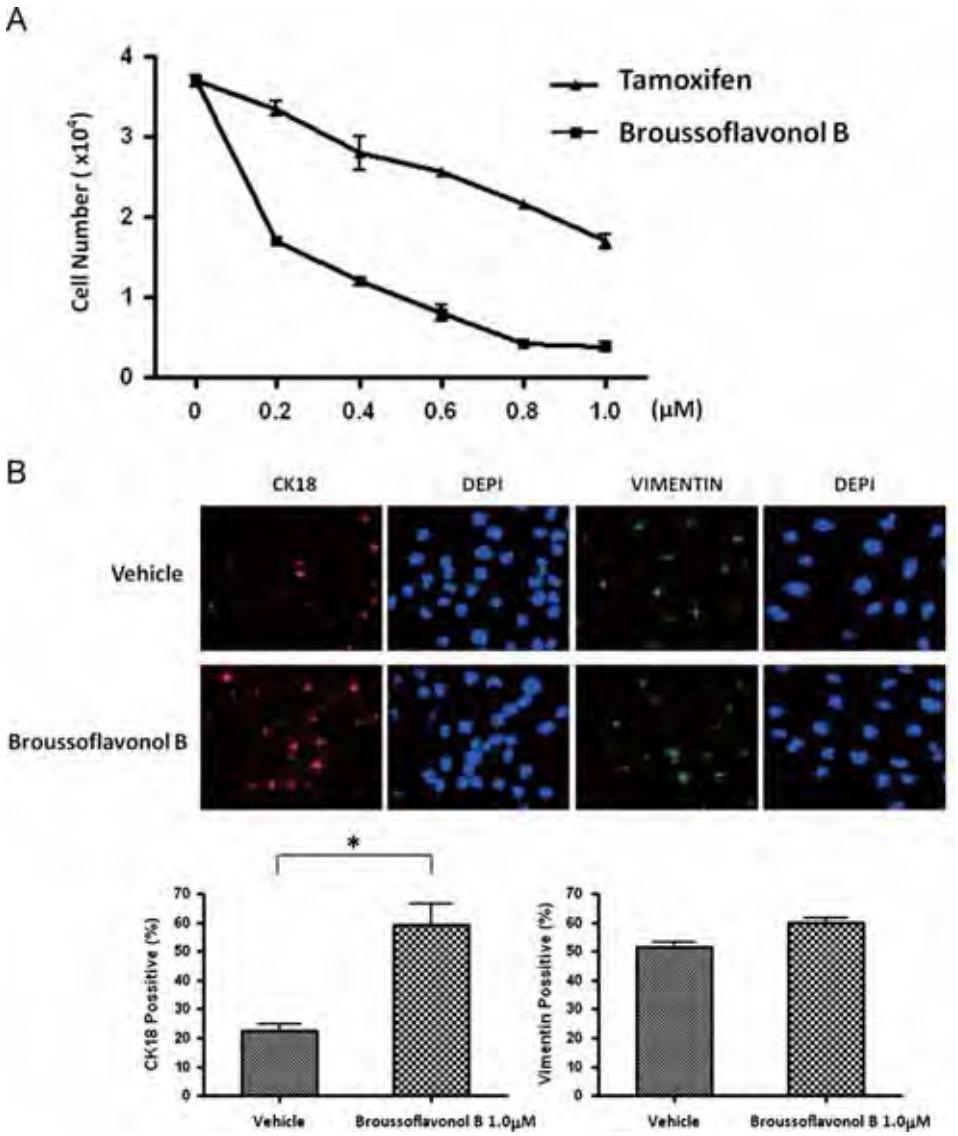

Fig. 7. Broussoflavonol B inhibits growth of ER-negative breast cancer stem/progenitor cells. (A) MDA-MB-231 cells were seeded onto Corning Ultra-Low Attachment 6-well plate and cultured in stem cell medium. Different concentrations of Broussoflavonol B or tamoxifen were added and incubated for seven days. Cells were counted using the ADAM automatic cell counter. Three dishes were used for each treatment and experiments were repeated three times. Each point represents mean ± S.D. of three independent experiments. (B) MDA-MB-231 cells were cultured in the stem cell medium for seven days. Indicated concentrations of Broussoflavonol B were added and incubated for another three days. Cells were collected and cytospined onto slides. Cytospined slides were stained with indirect immunofluorescent staining using anti-CK18 and vimentin antibodies (upper panels). Five hundred cells were assessed for vimentin or CK18 positivity under the fluorescent microscope and the percentage of cells positive for these markers were calculated (lower panels). The experiments were repeated three times. $^{*}P < 0.01$.

cells, the G_0/G_1 arrest of the cell cycle was accompanied with down-regulation of the growth-promoting protein c-Myc and induction of the cell cycle inhibitors including $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$. Thus, The G_0/G_1 phase arrest is also involved in Broussoflavonol B inhibitory function in growth of ER-negative MDA-MB-231 cells.

Furthermore, flow cytometric analysis using Annexin V/PI staining demonstrated that Broussoflavonol B could dose-dependently induced apoptosis in MDA-MB-231 cells. Caspases are the principal effectors of apoptosis involved in pathways such as caspase 8 regulated extrinsic and caspase 9-regulated intrinsic pathways. The caspase 9 pathway links mitochondrial damage to caspase activation, and serves as an index of damage in mitochondrial membrane function (Bao and Shi, 2007). In addition, the downstream member caspase 3 is an executor of DNA fragmentation (Bao and Shi, 2007). As expected, we observed that Broussoflavonol B treatment induced activation of caspases 8, 9 and 3. Thus, proteolytic processing of the initiator caspases as well as the executor caspase, and subsequent apoptosis contributed to growth inhibitory activity of Broussoflavonol B.

Accumulating evidence indicated that many types of cancer, including breast cancer, are initiated from cancer stem/progenitor cells (Liu et al., 2005; Charafe-Jauffret et al., 2009). These cancer stem/progenitor cells are resistant to most therapeutic approaches currently used (Dean, 2006; Diehn and Clarke, 2006; O'Brien et al., 2009; Hambardzumyan et al., 2006; Shafee et al., 2008). In this study, we showed that ER-negative breast cancer stem-like cells were resistant to anti-estrogen tamoxifen, consistent with the concept that cancer stem/progenitor cells are resistant to current cancer therapies. Thus, the development of novel drugs that are able to selectively attack the cancer stem cells is of the greatest priority. Recently a large scale screening was conducted to seek agents selectively kill epithelial cancer stem cells, and salnomycin was identified as a potent agent specifically targeting breast cancer stem cells (Gupta et al., 2009). More recently, dietary chemopreventive agents sulforaphane and benzyl isothiocyanate were

reported to be able to inhibit growth of breast cancer stem cells both in vitro and in vivo (Li et al., 2010; Kim et al., 2013). It is worth noting that we have previously reported that phenethyl isothiocyanate acted more potently than the "pure" antiestrogen ICI 182,780 to down-regulates ER-α36 expression and to inhibit breast cancer cell growth (Kang and Wang, 2010). Together with our current finding, these results suggested that down-regulation of ER-α36 and function is a novel approach to target breast cancer stem/progenitor cells.

In this study, we also found that Broussoflavonol B not only inhibited growth of ER-negative breast cancer stem-like cells but also induced differentiation of these cells, suggesting that differentiation induction may be one of the mechanisms by which Broussoflavonol B restricts growth of ER-negative breast cancer stem-like cells. Based on the cancer stem cell model, tumors are originated from malignantly transformed stem cells that are able to self-renew (Clarke et al., 2006). Thus, induction of cancer stem cell differentiation or "destemming" cancer stem cells provides a novel therapeutic option to eliminate cancer stem cells. Thus, our results demonstrated that induction of cancer stem-like cell terminal differentiation or "destemming" cancer stem cells is a feasible therapeutic approach to eradicate human breast cancer by eliminating cancer stem cells.

5. Conclusion

Our results demonstrated that Broussoflavonol B from the Paper Mulberry tree possesses potent anti-growth activity; inducing the arrest of the cell cycle and cell death in ER-negative breast cancer cells. Broussoflavonol B also effectively downregulates the steady state levels of ER-α36 and EGFR proteins, indicating that Broussoflavonol B acts like a selective estrogen receptor down-regulator (SERD) and a disruptor of the positive regulatory loop consisted of ER-α36 and EGFR in ER-negative breast cancer cell. More importantly, our results also indicated that Broussoflavonol B restricts growth of breast cancer stem-like cells. Thus, our results provide experimental evidence for the hypothesis that ER-α36 can serve as a target to develop novel and effective therapeutic approaches for ER-negative breast cancer.

Acknowledgements

We thank Perry Greg for his technical support for flow cytometry. This work was supported by Department of Defense grant DAMD 11-1-0497 and the Nebraska Tobacco Settlement Biomedical Research Program Awards LB-606 (Stem Cell 2011-13) and LB595 to Z.Y. Wang. This work was also supported by National Natural Science Foundation of China (31240031) to M. L. Wang.

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ANTICANCER RESEARCH *33*: 1873-1880 (2013)

Broussoflavonol B Restricts Growth of ER-negative Breast Cancer Stem-like Cells

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Abstract. *Accumulating experimental and clinical evidence has indicated that tumor-initiating or cancer stem-like cells are a sub-population of tumor cells capable of initiating and driving tumor growth, and cancer stem-like cells are resistant to most current cancer therapies, including chemo- and radiation therapy. More effective targeted-therapeutic approaches are urgently needed to eliminate cancer stem-like cells. Here, we report that broussoflavonol B, a chemical purified from the bark of the Paper Mulberry tree (broussonetia papyrifera), exhibited potent growth inhibitory activity towards estrogen receptor (ER)-negative breast cancer SK-BR-3 cells at sub-micromolar concentrations. Broussoflavonol B more potently inhibited growth and induced differentiation of stem-like SK-BR-3 cells-compared to the anti-estrogen tamoxifen. In addition, broussoflavonol B treatment also reduced the steady, state levels of the Human epidermal growth factor receptor-2 (HER2) and ER-α36, a variant of ER-α. Our results, thus, indicate that broussoflavonol B is a potent growth inhibitor of ER-negative breast cancer stem-like cells and provide a rationale for pre-clinical and clinical evaluation of broussoflavonol B for breast cancer therapy.*

Tumor-initiating, or cancer stem-like, cells are a sub-population of tumor cells capable of initiating and driving tumor growth. Accumulating experimental and clinical evidence supports the hypothesis that breast cancer arises from a sub-population of mammary stem/progenitor cells that possess the ability to self-renew (1-5). Al-Hajj *et al*. enriched a $CD44^+/CD24^{-/low}$ cell population from human breast cancer that displayed cancer stem/progenitor cell properties and was capable of forming tumors in immunocompromised mice with higher efficiency than in cells with alternative phenotypes (6).

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Key Words: Broussoflavonol B, estrogen receptor, cancer stem-like cells.

Later, aldehyde dehydrogenase (ALDH)-1 expression and its activity were identified to be a marker for breast cancer stem/progenitor cells; fewer ALDH1-positive tumor cells than $CD44^+/CD24^{-/low}$ tumor cells are required to generate tumors *in vivo* (7). Breast cancer with $ALDH1^{high}$ cancer stem-like cells are associated with more aggressive tumor phenotypes such as these with estrogen receptor negativity, high histological grade, Human epidermal growth factor receptor-2 (HER2) positivity, as well as poor prognosis (8).

Accumulating evidence has indicated that cancer stem-like cells are resistant to many current cancer therapies, including chemo- and radiation therapy as well as hormone therapy (9-13). This suggests that many cancer therapies, while killing the bulk of tumor cells, may eventually fail since they do not eliminate cancer stem-like cells that survive to regenerate new tumors. Thus, novel and effective therapeutic agents that target cancer stem-like cells are urgently needed.

Previously, we identified and cloned a 36-kDa variant of ER-α, ER-α36, that is mainly expressed at the plasma membrane and in the cytoplasm, and mediates non-genomic estrogen signaling (14, 15). ER-α36 lacks both transcription activation function domains AF-1 and AF-2 of the full-length 66 kDa ER-α (ER-α66), consistent with the fact that ER-α36 has no intrinsic transcriptional activity (15). ER-α36 is generated from a promoter located in the first intron of the *ER-α66* gene (16), indicating that ER-α36 expression is regulated differently from ER-α66, consistent with the findings that ER-α36 is expressed in specimens from ER-negative breast cancer and established ER-negative breast cancer cells that lack ER-α66 expression (17, 18). ER-α36 is highly expressed in ER-negative SK-BR-3 breast cancer cells and positively regulates HER2 expression in these cells (19). ER-α36 expression is required for maintenance of the ALDH1-positive stem-like SK-BR3 cells; knockdown of ER-α36 expression with the short hairpin RNA (shRNA) method dramatically reduced the population of ALDH1-positive cells (19). Thus, ER-α36-mediated signaling plays an important role in maintenance of ER-negative breast cancer stem-like cells, and down-regulation of ER-α36 expression may provide a novel approach to inhibit proliferation of ER-negative breast cancer stem-like cells.

Recently, we reported that several flavonoid derivatives purified from the bark of the Paper Mulberry tree (*Broussonetia papyrifera*) (L.) were able to down-regulate ER-α36 expression (20). In the present study, we examined the growth inhibitory activity of the most potent ER-α36 down-regulator broussoflavonol B (5,7,3',4'-tetrahydroxy-3-methoxy-6,8-diprenylflavone) from the bark of the Paper Mulberry tree on SK-BR-3 cells.

Materials and Methods

Chemicals and reagents. Broussoflavonol B (99.8% pure) was obtained from Beijing Shenogen Pharma Group (Beijing, China). Antibody to HER2 was purchased from Cell Signaling Technology (Danvers, MA, USA). Polyclonal antibody to ER-α36 was generated and characterized as described elsewhere (18). Antibodies to β-actin, CD10 (H-321), and cytokeratin-18 (DC-10) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. SK-BR-3 cells were purchased from the American type culture collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 1% antibiotic-antimycotic from Invitrogen (Carlsbad, CA, USA). Before experiments, cells were maintained in phenol red-free media with 2.5% charcoal-stripped FBS (Thermo Scientific Hyclone, Logan, UT, USA) for 24 h. All cells were maintained at 37˚C and in 5% CO_2 in a humidified incubator. The ALDEFLUOR kit (StemCell Technologies, Vancouver, BC, Canada) was used to examine the cell population with high ALDH1 enzymatic activity according to the manufacturer's instructions using a FACSCalibur flow cytometer (BD-Biosciences, San Jose, CA, USA).

Cell growth assay. Cells in phenol red-free medium were seeded into 35 mm dishes at 5×10^4 cells/dish. After 24 h, broussoflavonol B (0. 1 to 1 μM) or tamoxifen (0.1 to 1 μM) were added and cells incubated for another seven days. The vehicle dimethyl sulfoxide (DMSO) was used as a control. Cells were then trypsinized and counted using the ADAM automatic cell counter (Digital Bio, Soul, Korea). Three dishes were used for each concentration tested and the experiments were repeated three times.

For the growth assay of cancer stem-like cells, SK-BR-3 cells were seeded onto Corning Ultra-Low Attachment 6-well plates (Corning Incorporated, Corning, NY, USA) at 10,000 cells/ml and cultured in the stem cell culture medium: phenol-red free DMEM/F12 medium (Invitrogen) supplemented with 1X B27 (Invitrogen), 20 ng/ml epidermal growth factor (Sigma-Aldrich, St. Louis, MO, USA) and 20 ng/ml basic fibroblast growth factor (ProSpec, NJ, USA), 0.5 μg/ml hydrocortisone (Sigma). Different concentrations of broussoflavonol B or tamoxifen were added and cells were then incubated for seven days. Cells were then collected, washed with phosphate buffered saline (PBS) and trypsinized to dissociate cells, then counted using the ADAM automatic cell counter (Digital Bio) or examined for ALDH1 positive cells using the ALDEFLUOR kit.

Indirect immunofluorescent staining. Treated cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS and then permeabilized in 0.5% Triton X-100 for 15 min. After washing with PBS, cells were incubated for 1 h at room temperature with different primary antibodies followed by extensive washing with PBS. The cells were then incubated for over 1 h at room temperature with secondary antibody Alexa 488-conjugated rabbit anti-mouse (Molecular Probes, Carlsbad, CA, USA) diluted 1:100 in PBS. Cells were washed with PBS and mounted with 10 mg/ml 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) in aqueous mountant (Dako, Carpinteria, CA, USA) and photographed using a Zeiss fluorescence microscope (Carl Zeiss).

Western blot assay. Cells were washed with cold PBS twice and lysed with the radioimmunoprecipitation assay (RIPA) buffer containing 1% proteinase inhibitor cocktail solution and 1% phosphatase inhibitor cocktail solution (Sigma-Aldrich). The cell lysates were boiled for five minutes in sodium dodecyl sulfate (SDS) gel-loading buffer and separated on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with appropriate primary antibodies, which were then visualized with the corresponding secondary antibodies and ECL kit (Thermo Scientific, Rockford, IL, USA).

Cell differentiation assay. For the differentiation assay of cancer stem-like cells, SK-BR-3 cells were seeded onto Corning Ultra-Low Attachment 10 cm dishes (Corning Incorporated) at 10,000 cells/ml and cultured in the stem cell culture medium for seven days. Broussoflavonol B or tamoxifen were then added and cells were incubated for another three days. Cells were collected, washed with PBS and cytospinned onto slides. Cytospinned slides were stained with indirect immunofluorescent staining using antibodies to CD10 and CK18. Five hundred cells were assessed for CD10 or CK18 positivity under fluorescence microscopy (Nikon, Eclipss E600, Melville, NY, USA), and the percentage of cells positive for these markers was calculated.

Statistical analysis. Data are summarized as the means±standard deviation (S.D.) using GraphPad InStat software program. Statistical analysis was performed using paired-samples *t*-test, or ANOVA followed by the Student-Newman-Keuls testing and the significance was accepted for *p*-values less than 0.05.

Results

Broussoflavonol B inhibits proliferation of ER-negative SK-BR-3 breast cancer cells. Recently, we reported that broussoflavonol B (Figure 1A) was able to down-regulate ER-α36 expression in ER-positive MCF7 breast cancer cells (20). Since ER-α36 plays an important role in malignant growth in ER-negative breast cancer SK-BR-3 cells (19), we decided to test whether broussoflavonol B influences their growth. SK-BR-3 cells were incubated with different concentrations of broussoflavonol B or the classical anti-estrogen tamoxifen for seven days, and the numbers of surviving cells were counted. We found that broussoflavonol B potently inhibited growth of SK-BR-3 cells, while tamoxifen had no effect (Figure 1B), consistent with the fact that anti-estrogens have less or no effect on the growth of ER-negative breast cancer cells.

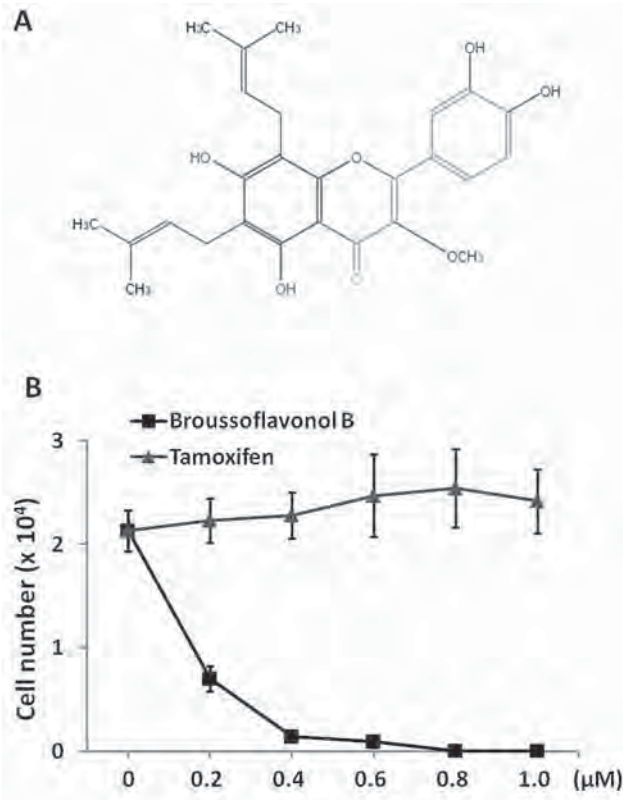

Figure 1. *Broussoflavonol B inhibits growth of estrogen receptor (ER)-negative SK-BR-3 breast cancer cells. A. The chemical structure of broussoflavonol B (5,7,3',4'-tetrahydroxy-3-methoxy-6,8-diprenylflavone). B. Broussoflavonol B inhibits growth of SK-BR-3 breast cancer cells. Cells maintained in phenol red-free medium with 2.5% charcoal-stripped fetal calf serum were treated with vehicle dimethyl sulfoxide (DMSO, 0), 0.2, 0.4, 0.6, 0.8 and 1.0 μM of broussoflavonol B or tamoxifen for seven days before cells were trypsinized and counted. Three dishes were used for each concentration in all of the experiments and all experiments were repeated three times. Each point represents the mean±S.D. of three independent experiments.*

Broussoflavonol B down-regulates expression of both ER-a36 and HER2 in SK-BR-3 cells. To probe the underlying mechanisms by which broussoflavonol B inhibits the growth of SK-BR-3 cells, we decided to determine whether broussoflavonol B influences ER-α36 expression in SK-BR-3 cells. Western blot analysis indicated that broussoflavonol B treatment potently down-regulated ER-α36 expression in a dose- and time-dependent manner (Figure 2A and B), whereas the anti-estrogen tamoxifen modestly increased the levels of ER-α36 expression in SK-BR-3 cells (Figure 2C). Previously, we found that a positive regulatory loop between ER-α36 and HER2 is critical for growth of SK-BR-3 cells; they positively regulate each other's promoter activity (19). We also found that HER2 expression was dramatically down-regulated in SK-BR-3 cells treated with broussoflavonol B (Figure 2D). Thus, our data indicate that disruption of the positive regulatory loop of ER-α36 and HER2 is one of the mechanisms by which broussoflavonol B inhibits growth of these cells.

Broussoflavonol B inhibits the growth of breast cancer stem/progenitor cells. Accumulating evidence has demonstrated that many types of cancer, including breast cancer, are initiated from cancer stem/progenitor cells (1-6). Breast cancer stem/progenitor cells are involved in resistance to chemo-and radiation therapies (9-13). Previously, we reported that ER-α36 expression is required for maintenance of the ALDH1-positive stem-like cells in SK-BR-3 cells; knockdown of ER-α36 expression with the short hairpin (sh) RNA method significantly reduced the population of ALDH1-positive SK-BR-3 cells (19). We decided to test the effects of broussoflavonol B on stem-like SK-BR-3 cells. With this aim, we cultured SK-BR-3 cells in a low-serum stem/progenitor cell culture medium and in ultralow-attachment dishes, which enriches the breast cancer stem-like cells. These stem-like cells were then treated with different concentrations of broussoflavonol B or tamoxifen for seven days and cell numbers were counted. We found that broussoflavonol B effectively inhibited the growth of these stem-like breast cancer cells while tamoxifen had a lesser effect (Figure 3A). We then tested the effects of broussoflavonol B on the ALDH1-positive SK-BR-3 cell population. ALDH expression or its activity has been used as a marker for breast cancer stem/progenitor cells (7). We first treated SK-BR-3 cells with 1 μM and 5 μM of broussoflavonol B or tamoxifen for seven days, and the ALDH1-positive cells from the remaining SK-BR-3 cells were analyzed, using the ALDEFLOUR kit and flow-cytometry. We found that treatment of SK-BR-3 cells with broussoflavonol B significantly reduced the population of ALDH1-positive cells while tamoxifen at 1 μM weakly but significantly increased the ALDH1-positive cell population (Figure 3B). These results indicate that the ALDH-high cells, *i.e.* breast cancer stem-like cells, are resistant to the widely used anti-estrogen tamoxifen, and broussoflavonol B acts as a potent inhibitor of these breast cancer stem-like cells.

Broussoflavonol B induces differentiation of breast cancer stem-like cells. Based on the cancer stem cell model, tumors originate from transformed stem cells that are able to self-renew and give rise to relatively differentiated cells (cancer progenitor cells) through asymmetric division, thereby forming heterogeneous cell populations found in a tumor (21). Thus, induction of cancer stem cell differentiation or de-stemming of cancer stem cells provides a novel theapeutic option to eliminate cancer stem cells. To examine whether broussoflavonol B also induces differentiation of breast cancer stem cells, we treated SK-BR-3 cells cultured in stem

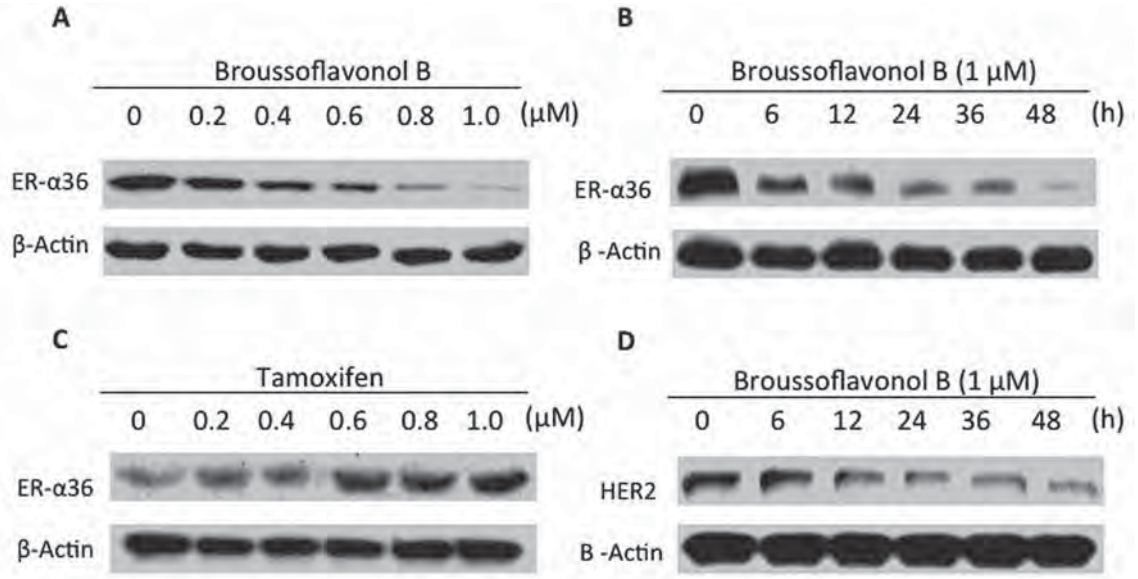

Figure 2. *Broussoflavonol B treatment down-regulates ER-α36 and human epidermal growth factor receptor-2 (HER2) expression. SK-BR-3 cells maintained in phenol red-free medium with 2.5% charcoal-stripped fetal calf serum were treated with dimethyl sulfoxide (DMSO, 0), and the indicated concentrations of broussoflavonol B for 12 h (A) or 1 μM of broussoflavonol B for the indicated hours (h) (B). Cell lysates were then subjected to western blot analysis with an antibody for ER-α36. C. Cells were treated with dimethyl sulfoxide (DMSO, 0) and the indicated concentrations of tamoxifen. Cell lysates were then subjected to western blot analysis with an antibody for ER-α36. D. Cells were treated with DMSO (0) and the indicated concentrations of broussoflavonol B. Cell lysates were then subjected to western blot analysis with an antibody for HER2. All membranes were stripped and re-probed with β-Actin antibody to ensure equal loading.*

cell culture medium with broussoflavonol B and tamoxifen for three days. The cells were then examined for expression of different differentiation markers including CK18 for luminal epithelial differentiation and CD10 for myoepithelial cell differentiation. We found that broussoflavonol B treatment significantly increased the number of cells positive for CK18 and modestly, but significantly increased the number of cells positive for CD10 (Figure 4), suggesting that broussoflavonol B is able to induce differentiation of ER-negative breast cancer stem-like cells mainly into the luminal epithelial lineage and differentiation induction may be one of the mechanisms by which broussoflavonol B restricts growth of ER-negative breast cancer stem-like cells. On the other hand, however, anti-estrogen tamoxifen had less or no effect on the differentiation of these cells (Figure 4), consistent with the previous hypothesis that cancer stem/progenitor cells are resistant to most cancer therapies (9-13).

Discussion

Anti-estrogen tamoxifen has been widely used to treat patients with ER-positive breast tumors, either as adjuvant therapy following surgery, or as first-line treatment for advanced disease. Tamoxifen was also approved as a chemopreventive agent for high-risk women who have a familial history of breast cancer. Although tamoxifen is effective as an adjuvant and chemopreventive agent for ER-positive breast cancer, the therapeutic efficacy of tamoxifen is dramatically reduced in ER-negative tumors. Thus, novel therapeutic agents are urgently needed for treatment of ER-negative breast cancer.

In this study, we investigated the growth-inhibitory potential of a flavonoid derivative broussoflavonol B from the Paper Mulberry tree that grows naturally in Asian and Pacific countries. Crude extracts from this plant exhibit various activities such as anti-platelet activity, inhibition of aromatizing enzymes, anti-oxidant, anti-microbial, anti-inflammatory, inhibition of PTP1B and cytotoxicity. Here, we demonstrated, to our knowledge, for the first time that broussoflavonol B potently inhibited growth of ER-negative SK-BR-3 breast cancer cells, presumably through down-regulation of ER-α36 and HER2 expression. We also demonstrated that broussoflavonol B induced differentiation of breast cancer stem-like cells and restricted the population of ALDH1-positive SK-BR-3 cells.

ER-α36 is highly expressed in ~40% of ER-negative breast cancer cases and its expression is significantly correlated with HER2 expression (18). Recently, we reported the existence of a positive feedback loop between HER2 and ER-α36 expression in SK-BR-3 cells; HER2 signaling activates the promoter activity of ER-α36 and ER-α36 signaling induces *HER2* promoter (19). In addition, we also found that ER-α36

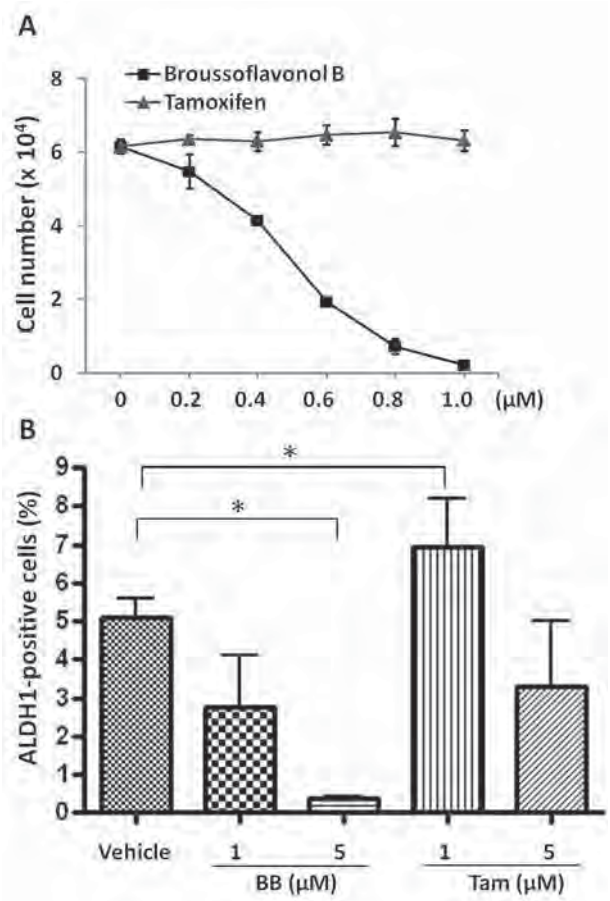

Figure 3. *Broussoflavonol B inhibits growth of ER-negative breast cancer stem-like cells. A: SK-BR-3 cells were maintained in stem cell culture medium and ultra-low attachment dishes and treated with dimethyl sulfoxide (DMSO, 0), the indicated concentrations of broussoflavonol B or tamoxifen for seven days. Cells were then trypsinized and counted. Three dishes were used for each concentration in all of the experiments and all experiments were repeated at least three times. Each point represents the mean±S.D. of three independent experiments. B: SK-BR-3 cells under regular culture conditions were treated with DMSO (0), or the indicated concentrations of broussoflavonol B (BB) or tamoxifen (Tam) for seven days. Cells were then trypsinized and analyzed with flow cytometry after staining with the fluorescent ALDEFLUOR kit. All experiments were repeated three times. Each column represents the mean±S.D. of three independent experiments. *p<0.05.*

is critical for maintenance of ALDH1-positive cancer stem-like SK-BR-3 cells (19), suggesting that down-regulation of ER-α36 expression may provide a novel therapeutic approach to treat ER-negative human breast cancer.

Recently, we reported that the selective estrogen receptor down-regulator (SERD)-ICI 182, 780 effectively down-regulated the expression of ER-α66 protein but increased the steady-state levels of the ER-α36 protein (22), suggesting that ICI 182, 780 only disrupts ER-α66 protein. Here, we found that broussoflavonol B potently down-regulated ER-α36 expression at sub-micromolar concentrations while tamoxifen had no effect, suggesting that broussoflavonol B is an ER-α36 down-regulator that may inhibit the non-genomic estrogen signaling mediated by ER-α36. In addition, we also found that broussoflavonol B down-regulated the steady-state levels of HER2 protein. Thus, our results strongly suggest that disruption of the positive feedback loop between HER2 and ER-α36 attenuates mitogenic signaling and restricts the malignant growth of ER-negative SK-BR-3 cells.

Accumulating evidence indicates that many types of cancer, including breast cancer, originate from and are maintained by a small population of cancer stem/progenitor cells (23). In this study, we showed that ER-negative breast cancer stem-like cells were also resistant to the anti-estrogen tamoxifen, consistent with the previous reports that cancer stem/progenitor cells are resistant to many current cancer therapies including chemo- and radiation therapy (9-13). However, broussoflavonol B inhibited growth of ALDH1-positive breast cancer stem-like cells and induced their differentiation.

According to the cancer stem cell model, tumors originate from cancer stem cells that are able to differentiate into non-cancer cells (1, 3). Thus, it was postulated that induction of cancer stem cell terminal differentiation or de-stemming of cancer stem cells may provide with a novel theapeutic option to eliminate cancer stem cells (24). Recently, it was shown that bone morphogenic protein-4 enhanced terminal differentiation, apoptosis and chemosensitization of colorectal cancer stem cells (25), suggesting the possibility of ligand-induced differentiation therapy. Here, we demonstrated that broussoflavonol B potently induced differentiation of ER-negative breast cancer stem-like cells mainly into the luminal epithelial lineage. We also found that broussoflavonol B was able to inhibit the growth of these stem-like cells, suggesting that broussoflavonol B may induce terminal differentiation of ER-negative breast cancer stem-like cells. Our results strongly indicate that induction of cancer stem-like cell terminal differentiation is a feasible theapeutic approach to eradicate human breast cancer by eliminating cancer stem cells.

Acknowledgements

We thank Perry Greg for his technical support for flow-cytometry. This work was supported by Department of Defense grant DAMD 11-1-0497 and the Nebraska Tobacco Settlement Biomedical Research Program Awards (LB-606 and LB692) to Z.Y. Wang.

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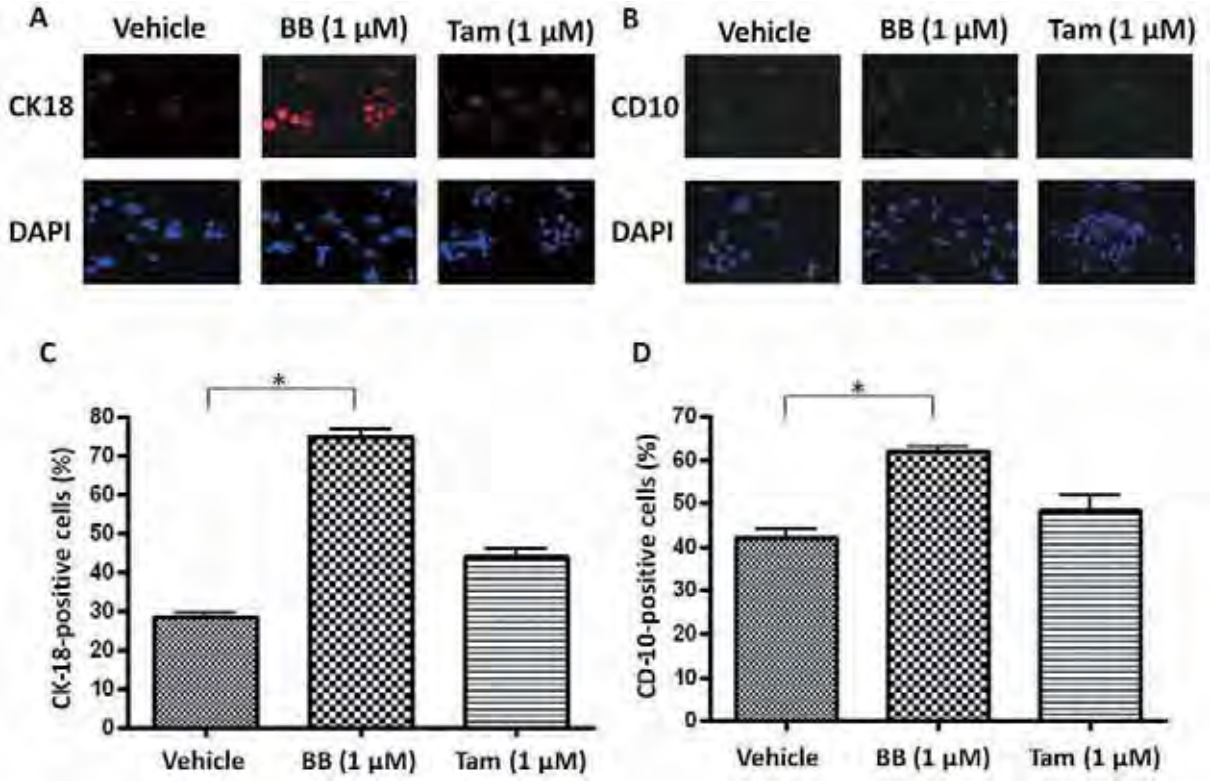

Figure 4. *Broussoflavonol B induces differentiation of ER-negative breast cancer stem-like cells. SK-BR-3 cells maintained in stem cells culture medium and ultra-low attachment dishes were treated with dimethyl sulfoxide (DMSO, vehicle), or 1 μM of broussoflavonol B (BB) or tamoxifen (Tam) for three days. Cells were then cytospinned onto slides, stained with antibodies against CK18 or CD10 and appropriate secondary antibodies, and photographed using a Zeiss fluorescence microscope. Five hundred cells were then assessed for CD10 and CK18 positivity under a fluorescence microscope and the percentage of cells positive for these markers was calculated. The experiment was repeated three times. Each column represents the mean±S.D. of three independent experiments. *p<0.01.*

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Received March 5, 2013
Revised April 10, 2013
Accepted April 11, 2013

OPEN ACCESS Freely available online

PLOS ONE

ER-α36-Mediated Rapid Estrogen Signaling Positively Regulates ER-Positive Breast Cancer Stem/Progenitor Cells

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Abstract

The breast cancer stem cells (BCSC) play important roles in breast cancer occurrence, recurrence and metastasis. However, the role of estrogen signaling, a signaling pathway important in development and progression of breast cancer, in regulation of BCSC has not been well established. Previously, we identified and cloned a variant of estrogen receptor α, ER-α36, with a molecular weight of 36 kDa. ER-α36 lacks both transactivation domains AF-1 and AF-2 of the 66 kDa full-length ER-α (ER-α66) and mediates rapid estrogen signaling to promote proliferation of breast cancer cells. In this study, we aim to investigate the function and the underlying mechanism of ER-α36-mediated rapid estrogen signaling in growth regulation of the ER-positive breast cancer stem/progenitor cells. ER-positive breast cancer cells MCF7 and T47D as well as the variants with different levels of ER-α36 expression were used. The effects of estrogen on BCSC's abilities of growth, self-renewal, differentiation and tumor-seeding were examined using tumorsphere formation, flow cytometry, indirect immunofluorence staining and *in vivo* xenograft assays. The underlying mechanisms were also studied with Western-blot analysis. We found that 17-β-estradiol (E2β) treatment increased the population of ER-positive breast cancer stem/progenitor cells while failed to do so in the cells with knocked-down levels of ER-α36 expression. Cells with forced expression of recombinant ER-α36, however, responded strongly to E2β treatment by increasing growth *in vitro* and tumor-seeding efficiency *in vivo*. The rapid estrogen signaling via the AKT/GSK3β pathway is involved in estrogen-stimulated growth of ER-positive breast cancer stem/progenitor cells. We concluded that ER-α36-mediated rapid estrogen signaling plays an important role in regulation and maintenance of ER-positive breast cancer stem/progenitor cells.

Citation: Deng H, Zhang X-T, Wang M-L, Zheng H-Y, Liu L-J, et al. (2014) ER-α36-Mediated Rapid Estrogen Signaling Positively Regulates ER-Positive Breast Cancer Stem/Progenitor Cells. PLoS ONE 9(2): e88034. doi:10.1371/journal.pone.0088034

Editor: Ming Tan, University of South Alabama, United States of America

Received September 30, 2013; **Accepted** January 2, 2014; **Published** February 18, 2014

Funding: This work was supported by the Nebraska Tobacco Settlement Biomedical Research Program Awards LB-606 (Stem Cell 2011-03) and LB595, and Department of Defense grant DAMD 11-1-0497. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Accumulating experimental and clinical evidence supports that breast cancer may arise from mammary stem/progenitor cells that possess the ability to self-renew [1–4]. Al-Hajj et al, enriched a $CD44^{+}/CD24^{-/low}$ cell population from human breast cancer that displayed cancer stem/progenitor cell properties and was capable of forming tumors in immuno-compromised mice with higher efficiency than cells with alternative phenotypes [1]. Later, aldehyde dehydrogenase (ALDH) 1 expression and/or its activity were identified to be a marker for breast cancer stem/progenitor cells; fewer ALDH1 positive tumor cells than $CD44^{+}/CD24^{-/low}$ tumor cells were required to generate tumors *in vivo* [5]. The breast cancers with $ALDH1^{high}$ cancer stem-like cells are often associated with more aggressive phenotypes such as estrogen receptor (ER) negativity, high histological grade, HER2 positivity, as well as poor prognosis [6].

Many signaling pathways involved in regulation of normal mammary stem cells including Hedgehog, Bmi-1, Wnt, NOTCH, HER-2, p53 and PTEN/Akt/β-catenin pathways play roles in breast cancer stem/progenitor cells [7–10]. However, the involvement of estrogen signaling, a major signaling pathway profoundly influences mammary carcinogenesis, in regulation of breast cancer stem/progenitor cells has not been well established, presumably since expression of estrogen receptor-α (ER-α) in breast cancer stem/progenitor cells remains controversial. It was reported that stem-like cells isolated from normal mammary gland and breast cancer tissues lack expression of the full-length ER-α [11,12]. However, Clarke *et.al* reported that ER-α is expressed in putative normal breast stem/progenitor cells enriched by the "side population" method [13]. Despite the fact that ER expression in mammary stem cells is not clear, the significance of estrogen signaling for normal development and growth of the mammary gland is well established by studies in human and animal, which was explained as though indirect paracrine pathways [14–17].

Previously, we identified and cloned a novel variant of ER-α, which has a molecular weight of 36-kDa. Thus, we have named it ER-α36 [18,19]. This ER-α variant differs from the original 66 kDa ER-α (ER-α66) because it lacks both transcriptional activation domains (AF-1 and AF-2) but retains the DNA-binding domain and partial ligand-binding domain [18]. It possesses a unique 27 amino acid stretch at the C-terminus to replace the last

138 amino acids of ER-α66. ER-α36 is mainly expressed at the plasma membrane and in the cytoplasm, and mediates non-genomic estrogen and antiestrogen signaling such as activation of the MAPK/ERK and PI3K/AKT signaling pathways [19,20].

Using a specific anti-ER-α36 antibody, we previously found that ER-α36 is expressed in specimens from both ER-positive and –negative breast cancer patients [19,21–23]. Recently, we reported that ER-α36-mediated estrogen signaling is critical for malignant growth of ER-negative breast cancer cells [24]. We also reported that ER-α36 expression is required for maintenance of the ALDH1-positive stem-like cells in ER-negative breast cancer SK-BR-3 cells [25], suggesting that ER-α36 is important in maintenance of the stem-like cells from ER-negative breast cancer. However, the function and underlying mechanisms of ER-α36-mediated estrogen signaling in regulation of the stem-like cells from ER-positive breast cancer are unknown.

Here, we show that ER-α36 is expressed in ER-positive breast cancer stem/progenitor cells, and ER-α36-mediated rapid estrogen signaling positively regulates ER-positive breast cancer stem/progenitor cells.

Materials and Methods

Reagents and Antibodies

The 17β-estradiol (E2β) was purchased from Sigma Chemical (St Louis, MO). The PI3K inhibitor LY294002 was from Tocris Bioscience (Ellisville, MO). The GSK-3β inhibitor IX, the AKT inhibitor IV, and the proteasome inhibitor MG132 were purchased from Calbiochem (San Diego, CA). The ER-α36 antibody was generated and characterized as described before [(19]. The β-actin antibody (1–19), anti-CK18 (DC-10) and anti-CD 10 (H-321) antibodies, anti-PCNA antibody (FL-261), the goat anti-mouse IgG-HRP, the goat anti-rabbit IgG-HRP and the donkey anti-goat IgG-HRP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The ER-α antibody (ERAb-16) was purchased from NeoMarkers (Fremont, CA). The antibodies for AKT, p-AKT (Ser473), GSK-3β27$\widetilde{C}_1$, p-GSK-3βD85E12, β-Catenin (D10AB) and p-β-Catenin (thr41/Ser45) were all purchased from Cell Signaling Technology (Danvers, MA). The ALDH1 antibody (#61194) was from BD Biosciences (San Jose, CA). PerCP-Cy^{TM}5.5 mouse anti-human CD44 (clone C26) and PE mouse anti-human CD24 (clone ML5) were purchased from BD Pharmingen (San Jose, CA). Anti-rabbit Alexa Fluor 488 antibody (A-11008) and anti-mouse Alexa Fluor 555 antibody (A-31570) were from Invitrogen (Carlsbad, CA).

Cell culture, Establishment of stable cell lines, and Growth assay

MCF7 and T47D cells were purchased from ATCC (Manassas, VA). The cells and their derivatives were cultured in Improved Minimal Essential Medium (IMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino-acids, 1% HEPES buffer, 1% antibiotic-antimycotic from Invitrogen (Carlsbad, CA) and 2 mg/ml bovine insulin (Sigma, St. Louis). All cells were maintained at 37°C and 5% CO2 in a humidified incubator.

MCF7 cells with forced expression of recombinant ER-α36 and with knocked-down levels of ER-α36 expression were established and characterized as described before [26,27]. To establish stable cell lines with knocked-down expression of ER-α36 from T47D cells, we constructed an ER-α36 specific shRNA expression vector by cloning the DNA oligonucleotides 5′-GATGCCAATAGG-TACTGAATTGATATCCGTTCAGTACCTATTGGCAT-3′ targeting the sequence in the 3′UTR of ER-α36 gene into the pRNAT-U6.1/Neo expression vector from GenScript Corp. Piscataway, NJ).

Briefly, T47D cells transfected with the empty expression vector and ER-α36 shRNA expression vector were selected with 500 μg/ml G418 for three weeks, and more than 20 individual clones from transfected cells were pooled, examined for ER-α36 expression with Western blot analysis and retained for experiments.

Tumorsphere formation, Self-renewal and Growth assays

To establish tumorspheres, cells were seeded onto Corning Ultra-Low Attachment 6-well plate (Corning Incorporated, CA) at 10,000 cells/ml and cultured seven days in the tumorsphere medium: phenol-red free DMEM/F12 medium (Invitrogen) supplemented with 1× B-27 (Invitrogen), 20 ng/ml epidermal growth factor (Sigma-Aldrich) and 20 ng/ml basic fibroblast growth factor (ProSpec, NJ), 0.5 μg/mL hydrocortisone (Sigma). Tumorspheres were collected, washed with PBS, and incubated with Trypsin-EDTA (0.25%/0.5 mM) for two minutes at 37°C to dissociated cells, and cells were counted using the ADAM automatic cell counter (Digital Bio, Korea).

To assess the self-renewal of the stem-like cells, tumorspheres were dissociated and cell number was determined. The cells from 1st generation of tumorspheres were seeded onto Ultra-Low Attachment 6-well plate at 5,000 cells/ml and cultured seven days in the tumorsphere medium to form 2nd generation tumorspheres. The cells were then passed once a week for 3rd and 4th generation tumorspheres. The number of tumorspheres and dissociated cells were counted using a Multisizer 3 Coulter Counter (Beckman Coulter, Brea, CA) and the ADAM automatic cell counter, respectively. For estrogen stimulation assays, tumorspheres were treated with 0.1 nM E2β or vehicle (ethanol) as a control. Three dishes were used for each group and all experiments were repeated three times.

Flow Cytometry Analysis

For CD44^{+}/CD24^{-} cell analysis, single cell suspension washed with cold PBS/1% BSA were incubated with PerCP-Cy^{TM}5.5 mouse anti-human CD44 and PE mouse anti-human CD24 in PBS/1% BSA for 30 minutes at 4°C. After incubation, the cells were washed twice in cold PBS/1% BSA and re-suspended in cold PBS/1% BSA for flow cytometry analysis.

DNA Transfection and Luciferase Assay

T47D and MCF7 cells were transfected with a p2×ERE-Luc reporter plasmid (a kind gift from Dr. Katarine Pettersson at Karolinska Institute, Sweden) using FuGene 6 transfection reagent (Roche Applied Science, Indianapolis, IN). Tumorspheres were transfected with electroporation using a pipette-type electroporator (MicroPorator MP-100, Digital Bio., Korean) as the manufacture recommended. All transfection included a cytomegalovirus-driven Renilla luciferase plasmid, pRL-CMV (Promega, Madison, WI) to establish transfection efficiency. Twenty-four hours after transfection, cells were treated with vehicle or 0.1 nM of E2β for 6, 12 and 24 hours. Cell extracts were prepared and luciferase activities were determined and normalized using the Dual-Luciferase Assay System (Promega, Madison, WI).

Western Blot Analysis

Cells were washed with cold PBS and lysed with the RIPA buffer containing 1% proteinase inhibitor cocktail solution and 1% phosphatase inhibitor cocktail solution (Sigma). The cell lysates were boiled for 5 minutes in sodium dodecyl sulfate (SDS) gel-loading buffer and separated on 10% SDS-PAGE gels. After

electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). The membranes were probed with appropriate primary antibodies and visualized with the corresponding secondary antibodies and the ECL kit (Thermo Scientific, Rockford, IL).

Indirect Immunofluorescence Assay

Cells were fixed in 4% paraformaldehyde for 10 minutes, then permeabilized in 0.1% Triton X-100 for 5 minutes, blocked in 1% BSA for 30 minutes, and then incubated with primary antibodies at 4°C overnight. Secondary antibodies, anti-rabbit Alexa Fluor 488 or anti-mouse Alexa Fluor 555 were then added and incubated for 1 hour at room temperature. Cells were washed with PBS and mounted with 10 mg/ml DAPI (4,6-diamidino-2-phenylindole dihydrochloride) (Sigma-Aldrich) in aqueous mountant (Dako, Carpinteria, CA) and photographed using a fluorescent microscope (Nikon, Eclipss E600).

Tumor Seeding Assays in Nude Mice

All animal procedures were approved by the Animal Care and Use Committee at the Creighton University and were performed in compliance with National Institutes of Health guidelines on the ethical use of animals. To assess tumor-seeding efficiency, cells in a serial dilution (1×10^2, 1×10^3, 1×10^4 and 1×10^5) were re-suspended in 0.1 ml of Matrigel and inoculated subcutaneously into the mammary fatpad of ovariectomized female nude mice (5–6 weeks old, strain CDI nu/nu, Charles River Breeding Laboratory). The mice were implanted with 0.35 mg/60-day slow-release 17β-estradiol pellets or placebos (Innovative Research of American, Sarasota, Florida) as controls. Mice were monitored twice a week for tumor growth. At the end of the experiments, the mice were euthanized, and the tumors were removed and weighed,

Statistical Analysis

Data were summarized as the mean ± standard deviation (S.D.) using GraphPad InStat software program. Statistical analysis was performed using paired-samples t-test, or ANOVA followed by the Student–Newman–Keuls testing and the significance was accepted for P values less than 0.05.

Results

Estrogen Expands the Population of ER-positive Breast Cancer Stem/Progenitor Cells

To examine the effects of estrogen signaling on growth of ER-positive breast cancer stem/progenitor cells, we used the well-characterized ER-positive breast cancer MCF7 and T47D cells as models. Both MCF7 and T47D cells were treated with or without 0.1 nM of E2β for five days. The $CD44^+/CD24^-$ stem-like cell populations in these cells were assessed with flow cytometry. We found that estrogen treatment significantly increased the $CD44^+/CD24^-$ cell population in both MCF7 and T47D cells (Figure 1A). We then cultured both MCF7 and T47D cells in the tumorsphere medium and under suspension conditions to form tumorspheres. We found that E2β treatment also increased the $CD44^+/CD24^-$ cell populations in tumorspheres from these cells (Figure 1A). We also found that E2β treatment markedly increased the size and number of the tumorspheres as well as the number of cells in the tumorspheres (Figure 1B and C). Our results thus suggested that estrogen signaling increases the population of ER-positive breast cancer stem/progenitor cells.

ER-α36 Plays an Essential Role in Mitogenic Estrogen Signaling of ER-positive Breast Cancer Stem/Progenitor Cells

We then examined ER-α36 function in the stem/progenitor cells derived from ER-positive breast cancer cells. MCF7 and T47D cells transfected with the empty expression vector (MCF7/V and T47D/V), MCF7 and T47D cells with knocked-down levels of ER-α36 expression (MCF7/Si36 and T47D/Si36), and MCF7/36 and T47D/36 cells with high levels of recombinant ER-α36 expression were used (Figure 2A). The $CD44^+/CD24^-$ cell populations in parental MCF7 and T47D cells as well as different variants treated with or without E2β for five days were assessed. We found that in the MCF7 and T47D cells that express high levels of ER-α36, MCF7/36 and T47D/36, the populations of $CD44^+/CD24^-$ cells were significantly increased compared to the control cells transfected with the empty expression vector, suggesting that ER-α36 is involved in positive regulation of ER-positive breast cancer stem/progenitor cells (Figure 2B). Estrogen treatment further increased the populations of $CD44^+/CD24^-$ stem-like cells in MCF7/36 and T47D/36 cells (Figure 2B). We also examined the $CD44^+/CD24^-$ cell populations in the tumorspheres formed by these cells treated with or without E2β. We found that in the tumorspheres formed by MCF736 and T47D/36 cells, the populations of $CD44^+/CD24^-$ cells were dramatically increased compared to the control MCF7/V and T47D/V cells, which was further increased by estrogen treatment (Figure 2B). However, we found that the cells with knocked-down levels of ER-α36 expression, MCF7/Si36 and T47D/Si36, exhibited decreased populations of the $CD44^+/CD24^-$ cell and weakly responded to estrogen treatment (Figure 2B).

We then tested the capability of these cells to form tumorspheres. We found that in the absence of estrogen, the MCF7/36 and T47D/36 cells formed more and bigger tumorspheres compared to the control cells transfected with the empty expression vector (Figure 2C, D). Estrogen treatment further increased the number and size of tumorspheres formed by these cells (Figure 2C, D). The MCF7/Si36 and T47D/Si36 cells, however, formed less and smaller size tumorspheres compared to the control cells, and these cells responded poorly to estrogen stimulation (Figure 2C, D). We also collected tumorspheres, dissociated cells of the tumorspheres and assessed cell number. We found that in the MCF7 and T47D cells with knocked-down levels of ER-α36 expression, the cell numbers in tumorspheres were dramatically decreased compared to the control cells and were not increased in response to estrogen treatment (Figure 2E). On the other hand, in the MCF7 and T47D cells with forced expression of ER-α36, the number of cells in tumorspheres were significantly increased compared to the control cells and were further increased in response to estrogen treatment (Figure 2E). These results strongly indicated that the ER-positive breast cancer cells with high levels of ER-α36 expression contain higher percentage of stem/progenitor cells, and ER-α36 plays a critical role in estrogen-stimulated growth of ER-positive breast cancer stem/progenitor cells.

ER-α36-mediated Estrogen Signaling Positively Regulates the Self-renewal of ER-positive Breast Cancer Stem Cells

According to the stem cell model, stem cells divide asymmetrically to maintain homeostasis of the stem cell pool, a process called self-renewal, while the growth of the bulk population relies on progenitor cells. To examine whether ER-α36-mediated estrogen signaling also influences the self-renewal of ER-positive breast cancer stem cells, we studied the tumorsphere formation of

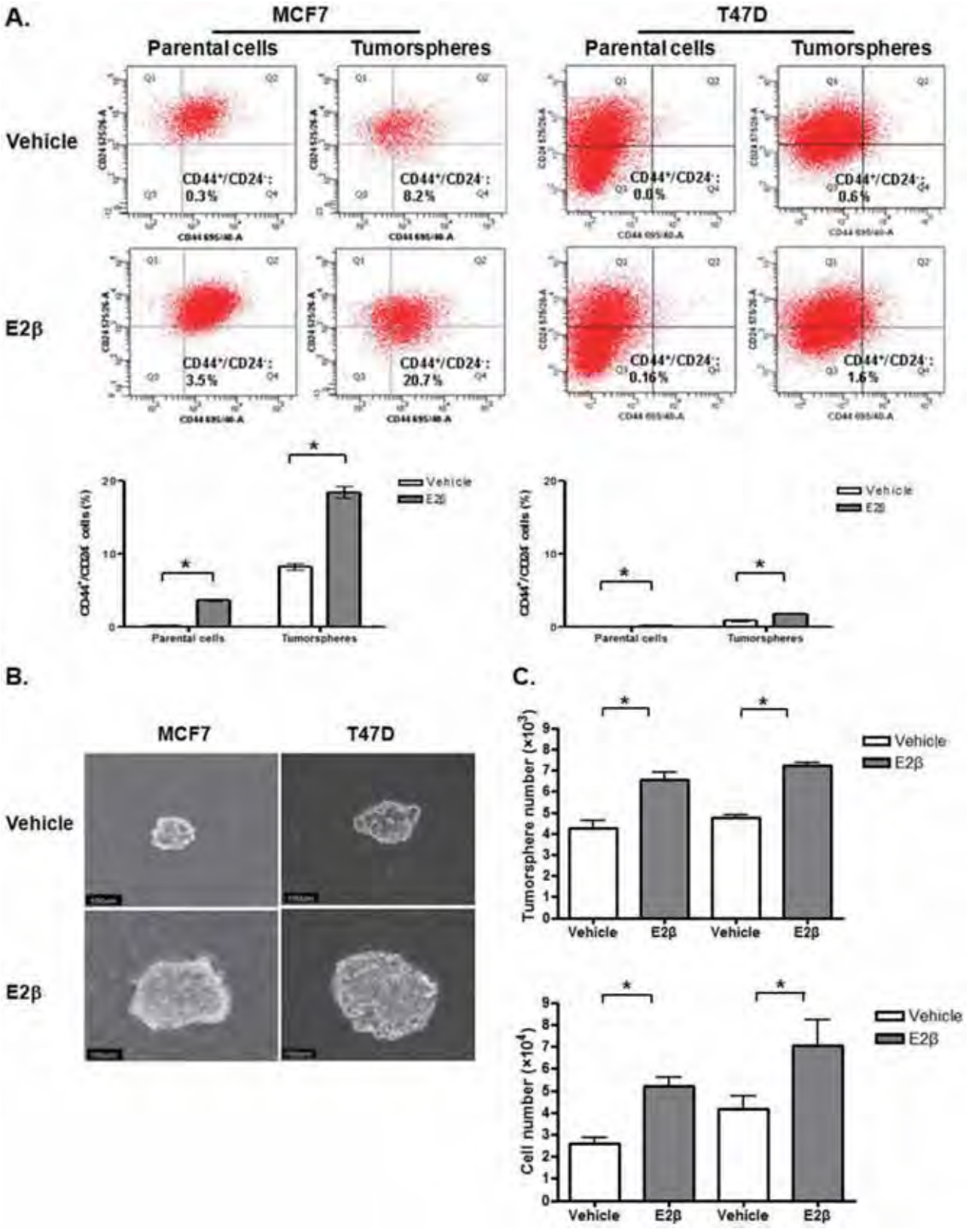

Figure 1. Estrogen expands the population of ER-positive breast cancer stem/progenitor cells. ER-positive breast cancer MCF7 and T47D cells were used. The tumorsphere formation assay and flow cytometry analysis of the $CD44^-$ and $CD24^+$ cells were used to assess the population of ER-positive breast cancer stem/progenitor cells. (A). Estrogen treatment increases the population of the $CD44^-/CD24^+$ cells in MCF7 and T47D cells.

The monolayer (parental) and tumorspheres of MCF7 and T47D cells were treated with vehicle (ethanol) or 0.1 nM of E2β for five days. The population of $CD44^-/CD24^+$ cells in these cells were analyzed after staining with fluorochrome-conjugated antibodies. The representative results are shown on the upper panel. Lower panel: the columns represent the means of three experiments; bars, SE. *, $P<0.05$ for vehicle treated cells vs cells treated with E2β. (B). Estrogen treatment increases the size of tumorspheres from MCF7 and T47D cells. A representative tumorsphere from MCF7 and T47D cells treated with vehicle or 0.1 nM E2β for seven days. (C). Estrogen treatment increases the number of tumorspheres and cells from dissociated tumorspheres derived from MCF7 and T47D cells. The columns represent the means of three experiments; bars, SE. *, $P<0.05$ for cells treated with vehicle vs cells treated with E2β.
doi:10.1371/journal.pone.0088034.g001

MCF7 and T47D cells as well as their derivatives with different levels of ER-α36 expression through serial passages in the absence or presence of estrogen. The cells were treated with vehicle or E2β at the time of each seeding. All viable cells were determined at the end of each passage and seeded for next passage for a total of four passages. We found that the MCF7 and T47D control cells transfected with the empty expression vector produced more tumorspheres in 2^{nd}, 3^{rd} and 4^{th} generations in the absence of estrogen while estrogen treatment further increased the number of tumorspheres in each generation (Figure 3A, B). Compared to the vector control cells, MCF7/36 and T47D/36 cells generated much more breast cancer stem/progenitor cells in 2^{nd}, 3^{rd} and 4^{th} generations of the self-renewal in the absence of estrogen, and estrogen treatment further enhanced growth of these cells (Figure 3A, B). In the absence and presence of estrogen, MCF7/Si36 and T47D/Si36 failed to generate more tumorspheres in each generation (Figure 3A, B). We also dissociated tumorspheres and determined the cell number. We found that cell numbers were increased more dramatically than the tumorsphere number in both cell lines, especially in the presence of estrogen (Figure 3C, D). Our results thus suggested that ER-α36-mediated estrogen signaling positively regulates the self-renewal of ER-positive breast cancer stem cells.

ER-α36-mediated Rapid Estrogen Signaling Enhances the Tumor-Seeding Efficiency of ER-positive Breast Cancer Stem/Progenitor Cells

Previously, MCF7-derived tumorsphere cells were reported to be more tumorigenic than the parental cells [28]. To assess the involvement of ER-α36-mediated estrogen signaling in tumor seeding efficiency of ER-positive breast cancer stem/progenitor cells, we evaluated the tumor forming potential of tumorsphere cells derived from MCF7 and T47D cells and their variants with different levels of ER-α36 expression using *in vivo* tumorigenic assay. We first enriched the breast cancer stem/progenitor cells using the tumorsphere formation assay. The tumorsphere cells were then injected in serial limiting dilution (1×10^2, 1×10^3, 1×10^4 and 1×10^5 cells) into the mammary fatpad of ovariectomized female nude mice that were implanted with 0.35 mg/60-day slow-release 17β-estradiol or placebo pellets. In the absence of estrogen, tumorsphere cells from MCF7/V cells formed tumors at efficiency of four out of six mice and five out of six mice injected with 1×10^4 and 1×10^5 cells, respectively while MCF7/Si36 cells generated small tumors in four out of six mice only when 1×10^5 cells was injected (Figure 4, Table S1). The tumorsphere cells from MCF7/36 cells, however, had high tumor initiating potential; forming tumors (5/6) at 1×10^3 cells in the absence of estrogen. In the presence of estrogen, however, tumorsphere cells from MCF7/36 cells exhibited potent tumor-initiating efficiency, and generated tumors at 100 cells while MCF7V cells required 1,000 cells to generated tumors. We also found that MCF7/Si36 cells generated smaller tumors than the tumors formed by the control MCF7/V cells (Figure 4, Table S1). Similar results were also obtained in tumorsphere cells derived from T47D cell variants (Figure 4, Table S1). Our results thus strongly suggested that ER-α36-mediated estrogen signaling enhances the tumor-initiating efficiency of ER-positive breast cancer stem/progenitor cells.

ER-α36-mediated Estrogen Signaling Induced Proliferation of Luminal Epithelial Lineage Specific ER-positive Breast Cancer Progenitor Cells

Breast cancer stem cells are able to differentiate into both luminal epithelial and myoepithelial cells [3]. We investigated the differentiation lineages of the stem cells derived from different MCF7 and T47D derivatives in the presence and absence of estrogen. Single cell suspensions from tumorspheres plated on collagen-coated coverslips or intact tumorspheres in suspension culture were treated with or without E2βfor five days, and the indirect immunofluoresces assay was performed to determine the effects of estrogen on differentiation lineages of these cells using cytokeratin 18 (CK18) for luminal epithelial cells and CD10 for myoepithelial cells. We found that tumorsphere cells plated on collagen-coated coverslips were fully differentiated into either luminal epithelial or myoepithelial lineages, and estrogen treatment had less or no effect on the differentiation (Figure S1), suggesting that estrogen treatment was unable to influence differentiation induced by cell attachment. We then assessed the effects of E2β on the spontaneous differentiation occurred in tumorspheres under suspension culture. In tumorspheres formed by MCF7 cells, we found that estrogen treatment increased the population of the cells that were stained positive for CK18 but without effect on the cells positive for CD10 (Figure 5A). We also found that estrogen treatment increased more number of cells expressing CK18 in MCF7/36 cells compared with MCF7/V cells (Figure 5A) while estrogen only slightly increased CK18 positive cells in MCF7/Si36 cells. Similar results were also observed in T47D cell variants; T47D/Si36 cells failed to respond to estrogen (Figure 5B).

To further examine whether estrogen treatment induces differentiation of breast cancer stem cells or increases proliferation of breast cancer progenitor cells that were in luminal epithelial lineage, we tested if the cells stained positive for CK18 were still proliferative. Indirect immunofluorescence staining was performed to examine the co-expression of CK18 and the proliferating cell nuclear antigen (PCNA), a marker for cell proliferation. We found that in MCF7 cells, the number of both PCNA and CK18 positive cells was low in the absence of estrogen. After estrogen treatment, however, the number of cells co-expressing both PCNA and CK18 was markedly increased (Figure 5C), indicating estrogen stimulates proliferation of luminal epithelial lineage specific breast cancer progenitor or intermediate cells.

The PI3K/AKT/GSK3β/β-catenin Signaling Pathway is Involved in ER-α36-mediated Mitogenic Estrogen Signaling of ER-positive Breast Cancer Stem/Progenitor Cells

We also investigated the underlying mechanism of ER-α36-mediated estrogen signaling in ER-positive breast cancer stem/progenitor cells. We treated tumorspheres formed by MCF7 and T47D cells with E2β and performed Western blot analysis using

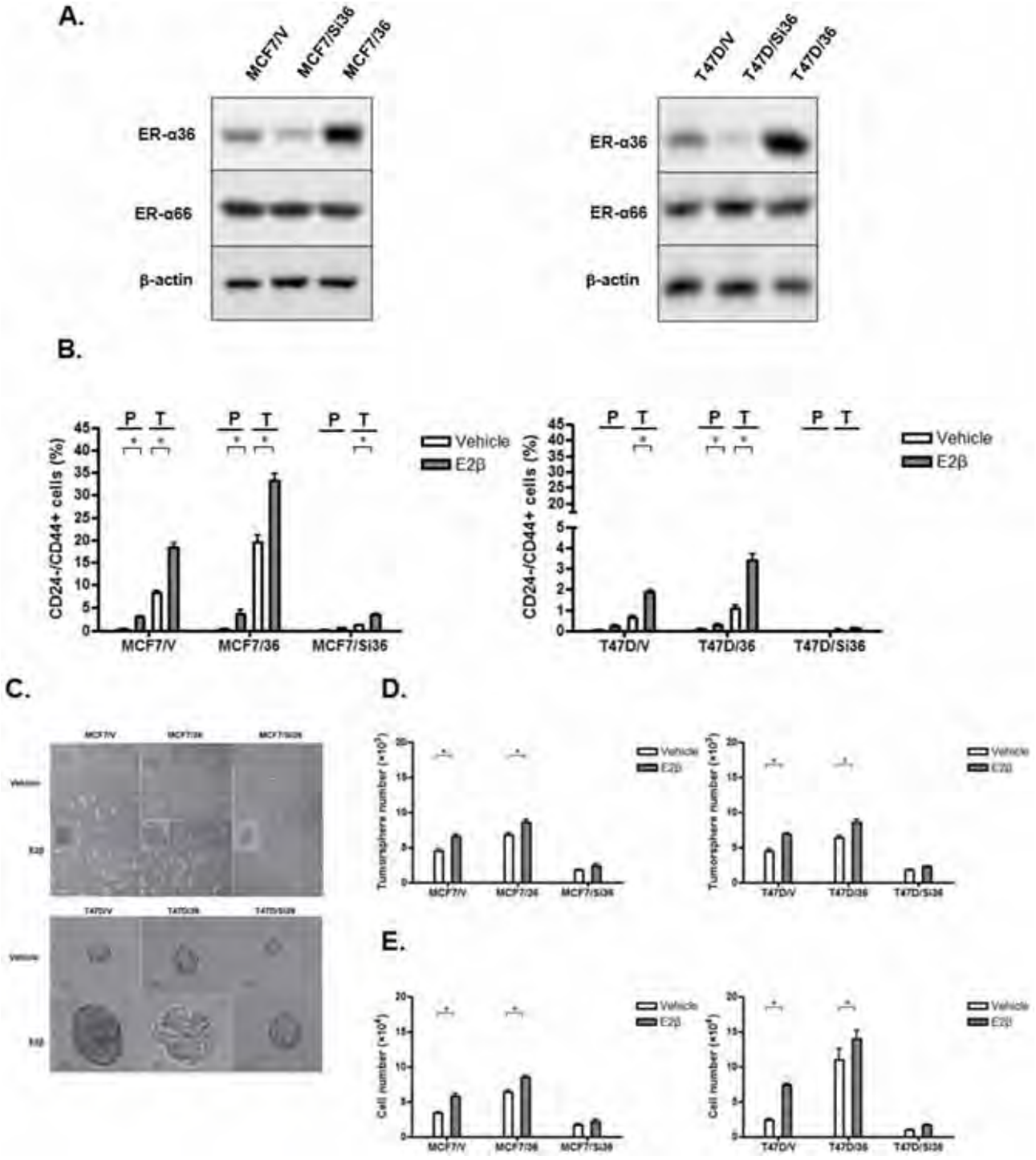

Figure 2. ER-α36-mediated rapid estrogen signaling positively regulates ER-positive breast cancer stem/progenitor cells. (A). Western blot analyses of ER-α36 expression in different MC7 and T47D cell variants; control cells (MCF7/V and T47D/V: cells transfected with the empty expression vector); cells with forced expression of ER-α36 (MCF7/36 and T47D/36: cells transfected with a ER-α36 expression vector); and ER-α36 expression knocked-down cells (MCF7/Si36 and T47D/Si36. (B). ER-α36-mediated estrogen signaling increases the population of the $CD44^{-}/CD24^{+}$ cells. The monolayer (parental, P) and tumorspheres (T) of MCF7 and T47D variants were treated with vehicle (ethanol) or 0.1 nM of E2β for five days. The population of $CD44^{-}/CD24^{+}$ cells in these cells were analyzed after staining with fluorochrome-conjugated antibodies. The columns represent the means of three experiments; bars, SE. *, $P<0.05$ for vehicle treated cells vs cells treated with E2β. (C). ER-α36-mediated estrogen signaling positively regulates the size and number of tumorspheres from MCF7 and T47D cells. Representative tumorspheres from MCF7 and T47D cell variants treated with vehicle or 0.1 nM E2β for seven days. Scale bar = 100 μm. (D). The numbers of tumorspheres and cells from dissociated tumorspheres of different cell variants were determined. The columns represent the means of three experiments; bars, SE. *, $P<0.05$ for cells treated with vehicle vs cells treated with E2β.
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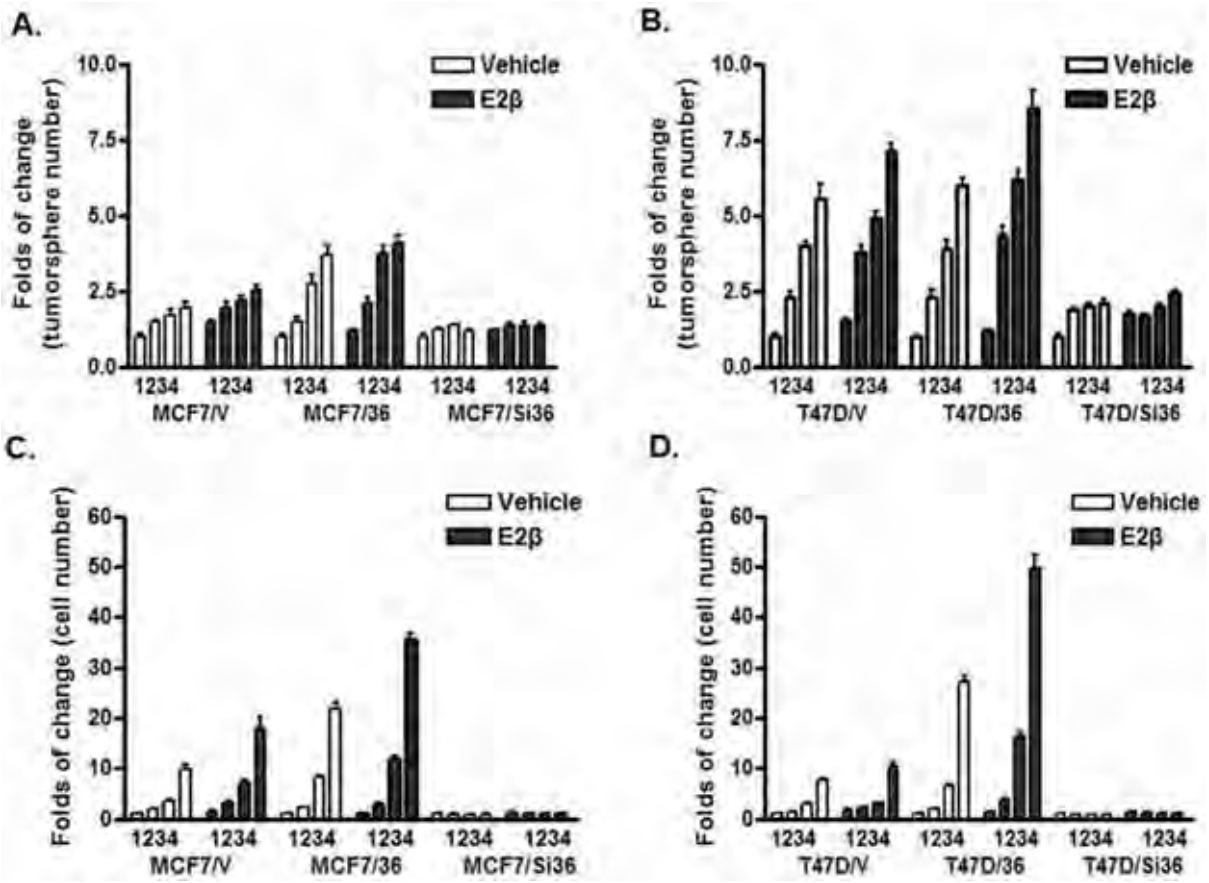

Figure 3. ER-α36-mediated estrogen signaling stimulates the self-renewal of ER-positive breast cancer stem cells. Long-term expansion of MCF7 (A.C) and T47D (B, D) variant cells in the presence of vehicle (ethanol) or 0.1 nM of E2β. The cells from tumorspheres were passed once a week for four generations. The numbers of tumorspheres and cells from dissociated tumorspheres were determined. The numbers of tumorspheres and cells from tumorspheres from the control cells transfected with the empty expression vector and treated with vehicle were arbitrarily set as 1. Three dishes were used for each group and the experiments were repeated three times. The columns represent the means of three experiments; bars, SE.
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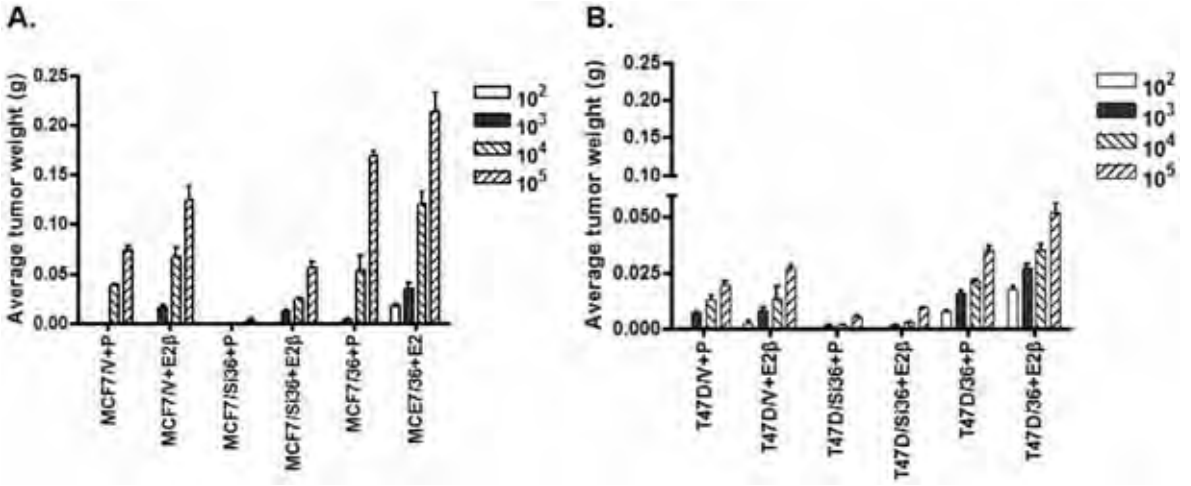


Figure 4. ER-α36-mediated estrogen signaling enhances the tumor-seeding efficiency of ER-positive breast cancer stem/progenitor cells. Different variants of MCF7 and T47D cells at limited dilutions were implanted in the mammary fatpad of the ovariectomized female mice supplemented with estrogen or placebo pellets. The tumor-seeding efficiency was examined by measurement of tumor weight. The data represent the mean ± SE observed in six mice in each group.
doi:10.1371/journal.pone.0088034.g004

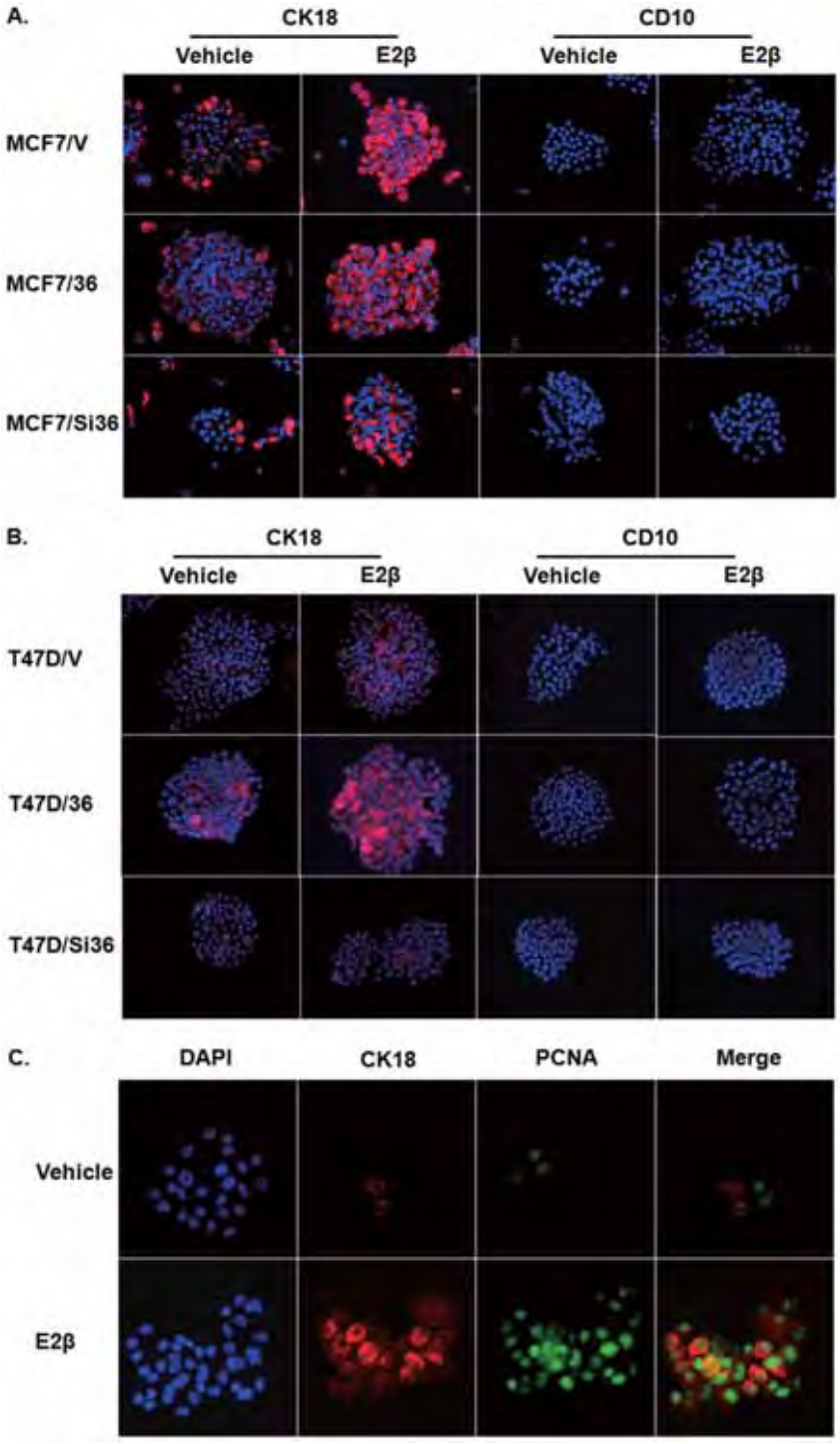


Figure 5. ER-α36-mediated estrogen signaling induced proliferation of luminal epithelial lineage specific ER-positive breast cancer progenitor cells. (A. B). Indirect Immunofluorescent staining for CK18 (red) or CD10 (red) in variants derived from MCF7 and T47D cells treated with vehicle or E2β. DAPI (blue) was used to stain the nuclear region. (C). Indirect Immunofluorescent staining for CK18 (red) or PCNA (green) in MCF7 cells treated with vehicle or E2β. DAPI (blue) was used to indicate the cell nuclei.
doi:10.1371/journal.pone.0088034.g005

various phosphorylation specific for the AKT, GSK-3β and β-catenin. We found that estrogen induced the activation of the PI3K/AKT/GSK3β/β-catenin signaling pathway in ER-positive breast cancer stem/progenitor cells, which was attenuated by the AKT inhibitor (Figure 6A). We then included chemical inhibitors for the PI3K, AKT and GSK3β during estrogen stimulation and found that inhibition of the PI3K, AKT and GSK3β attenuated estrogen-stimulated growth of the stem/progenitor cells (Figure 6B).

However, in the tumorspheres derived from MCF7/Si36 and T47D/Si36, estrogen failed to induce the AKT phosphorylation (Figure 6C). Our results thus indicated that the PI3K/AKT/GSK3β/β-catenin signaling pathway is involved in ER-α36-mediated mitogenic estrogen signaling of ER-positive breast cancer stem/progenitor cells.

The Expression and Genomic Function of ER-α66 are Down-regulated in ER-positive Breast Cancer Stem/Progenitor Cells

Since the expression and potential function of ER-α66 in the breast cancer stem/progenitor cells remains controversial, we decided to study the expression pattern and possible function of ER-α66 in tumorsphere cells derived from MCF7 and T47D cells that express high levels of endogenous ER-α66.

To assess the expression levels of ER-α66 and ER-α36 in ER-positive breast cancer stem/progenitor cells, we performed Western blot analysis with cell lysates from tumorspheres. We found that the expression levels of ER-α36 protein were dramatically increased in tumorspheres from MCF7 and T47D cells while ER-α66 expression was down-regulated compared to parental cells (Figure 7A). In addition, we also found that the expression levels of ALDH1 and the basal levels of the AKT and GSK3β phosphorylation were markedly increased in tumorspheres (Figure 7A). The expression levels of growth receptors EGFR and HER2 were also increased in tumorspheres (Figure 7A). When the tumorspheres derived from MCF7 and T47D cells were treated with MG132, a proteasome inhibitor, the steady state level of ER-α66 protein was dramatically increased in both parental cells and tumorshphere cells (Figure 7B), suggesting that degradation of ER-α66 protein by the proteasome system is involved in regulation of the steady state levels of ER-α66, which was enhanced in ER-positive breast cancer stem/progenitor cells. We then examined the expression patterns of ER-α66 and 36 in parental and tumorsphere cells using the indirect immunofluorescence staining. We found that ER-α36 is expressed at the plasma membrane and in the cytoplasm of both parental and tumorsphere cells (Figure 7C). ER-α66, however, exhibited a predominant nuclear staining in the parental MCF7 and T47D cells while a weak cytoplasm staining was also observed in T47D cells. In tumorsphere cells, ER-α66 was mainly expressed in the cytoplasm (Figure 7C), indicating a great portion of ER-α66 protein was redistributed to the cytoplasms of ER-positive tumorsphere cells. When the parental MCF7 and T47D cells, and their tumorsphere cells were transfected with a ERE containing luciferase reporter plasmid and treated with or without estrogen, we found that estrogen-induced transcription activities of ER-α66 were dramatically reduced in tumorsphere cells compared to parental cells (Figure 7D), indicating the genomic estrogen signaling mediated by ER-α66 is attenuated in ER-positive breast cancer stem/progenitor cells.

The luminal compartment of mammary gland could be separated into ER-α66 positive and negative cells. The ER-α66 positive luminal cells express prolactin and progesterone receptor, and more luminal cytokeratins than ER-α66 negative luminal cells [11]. MCF7 cells were cultured in suspension culture for three and seven days to form tumorspheres. Indirect immunofluorescence staining was performed to examine the expression of CK18 and ER-α66 in cells from the tumorspheres. We found that in the cells cultured for three days, CK18 was highly expressed, and ER-α66 was expressed mainly in the cell nucleus (Figure S2). In the tumorspheres cultured for seven days, however, a great portion of ER-α66 was redistributed from the cell nucleus to the cytoplasm and the signals for CK18 was diminished (Figure S2), indicating a correlation between cytoplasmic distribution of ER-α66 and downregulation of cells expressing CK18. Taken together, our results strongly suggested that ER-α66-mediated genomic estrogen signaling is important in cell differentiation, which is attenuated in ER-positive breast cancer stem/progenitor cells presumably through re-distribution and down-regulation of ER-α66 protein.

ER-α36 Expression is Positively Correlated to ALDH1 Expression in Specimens from Breast Cancer Patients

To further determine if ER-α36 is involved in positive regulation of breast cancer stem/progenitor cells *in vivo*, we examined the expression correlation of ER-α36 with ALDH1. We examined ER-α36 expression in sixty-eight specimens from breast cancer patients with the immunohistochemistry (IHC) assay and found that 34 out of 68 cases (50%) exhibited ER-α36 expression, predominantly in a cytoplasmic and membranous pattern (Figure S3, Table 1). The mean percentage of the ER-α36-positive cells was 53% and the majority of the cases showed moderate to strong ER-α36 staining. ALDH1 was detected in 30 cases (44%), 20 of which co-expressed ER-α36. There was a positive correlation between ER-α36 and ALDH1 expression ($P<0.01$, $x^2 5.96$). ER-α66 was expressed in 32 cases (47%), there was no correlation between ER-α66 and ALDH1 expression. These results suggested that ER-α36-mediated rapid estrogen signaling plays an important role in regulation of breast cancer stem/progenitor cells *in vivo*.

Discussion

In this study, the breast cancer stem/progenitor cells enriched from ER-positive breast cancer MCF7 and T47D cells were used as models to investigate their responses to estrogen. Here, we demonstrated that ER-α36-mediated rapid estrogen signaling plays an important role in maintenance and positive regulation of ER-positive breast cancer stem/progenitor cells. We showed that estrogen treatment expanded the population of breast cancer stem/progenitor cells and also stimulated the self-renewal of breast cancer stem cells, both of which were mediated by ER-α36. Knockdown of ER-α36 expression diminished tumor-seeding efficiency of ER-positive breast cancer stem/progenitor cells. We also showed that ER-α36 expression was markedly increased in the stem/progenitor cells enriched from ER-positive breast cancer cells accompanied by high levels of ALDH1, EGFR and HER2 as well as high levels of AKT and GSK3β phosphorylation. Finally, we presented evidence to indicate that the ER-α (ER-α66), was re-distributed outside of the cell nuclei, and its expression and genomic transcription activity were attenuated in ER-positive breast cancer stem/progenitor cells.

It is increasingly recognized that breast cancer has a population of cancer stem/progenitor cells that maintains tumor growth [29,30]. However, the function and underlying mechanisms of estrogen signaling in regulation of breast cancer stem/progenitor cells are not clear. Mammary stem cells of human and mouse are highly responsive to estrogen signaling, although they usually show a receptor negative phenotype for ER-α and PR [31,32]. A paracrine signaling model was proposed to explain the effects of estrogen signaling in mammary stem/progenitor cells [16,17]. Here, we demonstrated, for the first time, that estrogen positively regulated ER-positive breast cancer stem/progenitor cells via ER-α36-mediated rapid signaling pathway.

Expression of the full-length ER-α66 in the stem-like cells isolated from normal mammary gland and breast cancer tissues is controversial [11–13]. Here, using the well-established ER-positive breast cancer cells, we demonstrated that ER-α66 protein was re-distributed from the cell nucleus to the cytoplasm and was

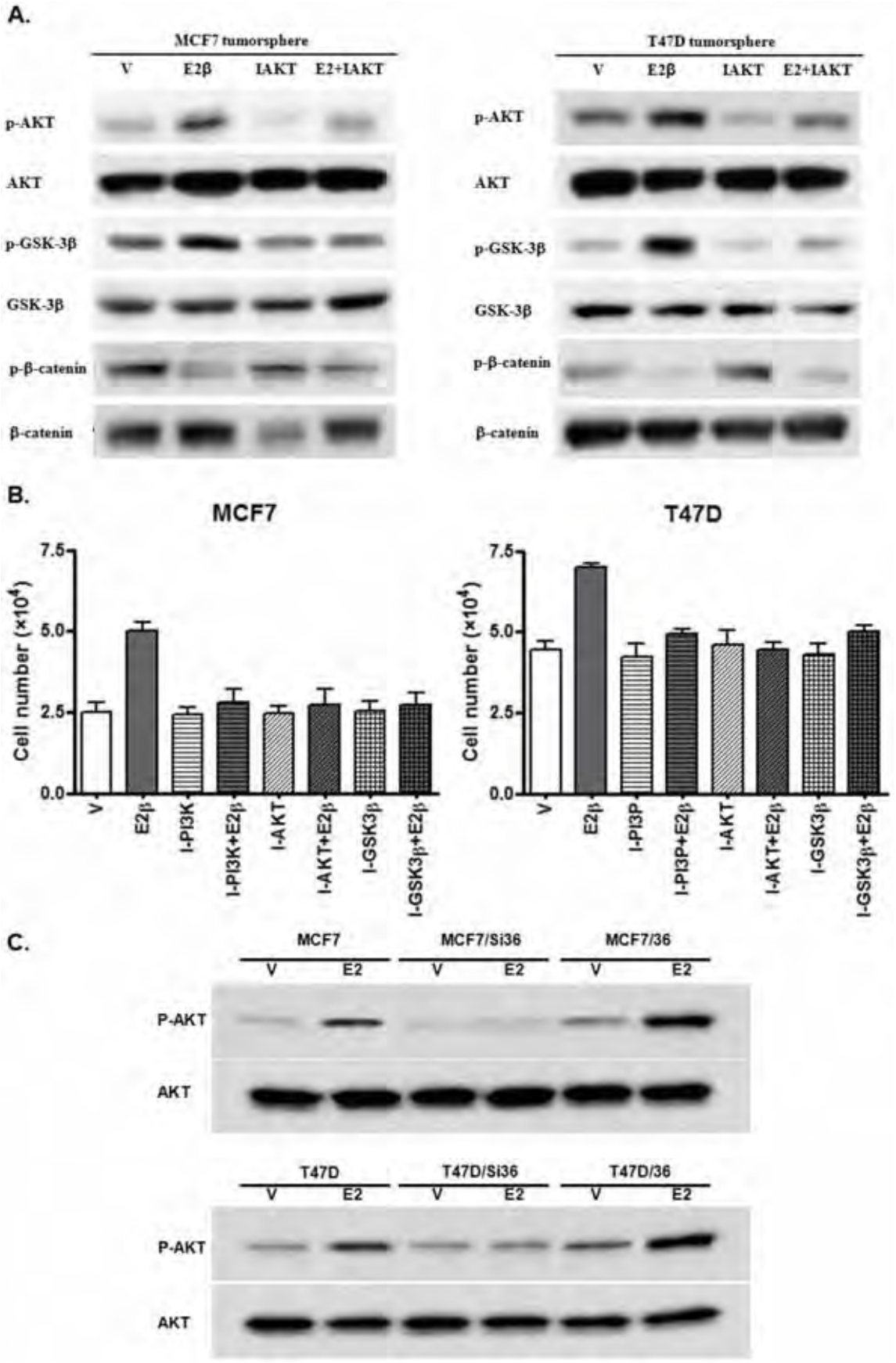

A.
MCF7 tumorsphere
V
E2β
IAKT
E2+IAKT
p-AKT
AKT
p-GSK-3β
GSK-3β
p-β-catenin
β-catenin
T47D tumorsphere
V
E2β
IAKT
E2+IAKT
p-AKT
AKT
p-GSK-3β
GSK-3β
p-β-catenin
β-catenin
B.
MCF7
Cell number (×10⁴)
7.5
5.0
2.5
0.0
V
E2β
I-PI3K
I-PI3K+E2β
I-AKT
I-AKT+E2β
I-GSK3β
I-GSK3β+E2β
T47D
Cell number (×10⁴)
7.5
5.0
2.5
0.0
V
E2β
I-PI3P
I-PI3P+E2β
I-AKT
I-AKT+E2β
I-GSK3β
I-GSK3β+E2β
C.
MCF7
MCF7/Si36
MCF7/36
V
E2
V
E2
V
E2
P-AKT
AKT
T47D
T47D/Si36
T47D/36
V
E2
V
E2
V
E2
P-AKT
AKT

Figure 6. The PI3K/AKT/GSK3β/β-catenin signaling pathway is involved in ER-α36-mediated mitogenic estrogen signaling of ER-positive breast cancer stem/progenitor cells. (A). Western blot analysis of the cell lysates from tumorspheres derived from MCF7 and T47D cells treated with ethanol (V); 0.1 nM of E2β; the AKT inhibitor IV (10 μM), IAkt; and E2β+IAkt, using indicated antibodies. (B). The effects of different inhibitors of the PI3K/AKT/GSK3β pathway on estrogen-stimulated growth of ER-positive breast cancer stem/progenitor cells. Tumorspheres of MCF7 and T47D cells were treated with vehicle, E2β alone or E2β together with the PI3K inhibitor LY294002 (10 μM), the GSK-3β inhibitor IX (10 μM), the AKT inhibitor IV (10 μM). After seven days, cell numbers from dissociated tumorspheres were determined. The columns represent the means of three experiments; bars, SE.
doi:10.1371/journal.pone.0088034.g006

destabilized presumably through the proteasome degradation system in ER-positive breast cancer stem/progenitor cells. As a result, the transcription activity of ER-α66 was attenuated in these cells. Thus, although ER-positive breast cancer stem/progenitor cells may retain ER-α66 expression, its function in genomic estrogen signaling may be diminished through redistribution and destabilization of the protein.

Previously, it was reported that ER-α66 positive luminal cells form a differentiated luminal compartment that express more luminal cytokeratins than ER-α66 negative luminal cells in mammary gland [17]. ER-α66 is often co-expressed with GATA3 in breast tumors and breast cancer cell lines [33–35]. GATA3 is a critical regulator of luminal differentiation that maintains the differentiation of the luminal cells in the mammary gland [36,37]. Our finding here that redistribution and down-regulation of ER-α66 were associated with decreased number of cells positive for CK18 in tumorspheres from ER-positive breast cancer cells highlighted an important role of ER-α66 in differentiation of luminal epithelial cells.

Here, we found that estrogen treatment increased both the numbers and sizes of tumorspheres from the ER-positive breast cancer cells, suggesting estrogen treatment expanded the pool of ER-positive breast cancer stem/progenitor cells via ER-α36-mediated signaling. Stem cells maintain self-renewal and differentiation in two ways: asymmetric and symmetric cell division [38,39]. Accumulating evidence suggested that dysregulation of asymmetric stem cell division is one of the reason for stem cell transformation [38,39]. However, the mechanisms by which stem cells adapt symmetric division have not been fully understood. Cicalese et al. reported that breast cancer stem cells derived from ERBB2/HER2 transgenic mice exhibited an increased frequency of symmetric self-renewing cell divisions and implicated p53 is a master regulator of this process [40]. Here, we found that in the presence of estrogen, ER-positive breast cancer cells with forced expression of ER-α36 increased the populations of breast cancer stem cells as evidenced by increased sizes and numbers of tumorspheres formed by these cells. However, cells with knocked-down levels of ER-α36 expression failed to increase the populations of stem/progenitor cells in response to estrogen while still retained the ability of the self-renewal. Since there are no specific markers to differentiate breast cancer stem, progenitor, and intermediate cells (non-stem proliferative cells), it is difficult to determine which cell populations that estrogen stimulates. However, the results that estrogen treatment increased both size and number of tumorspheres formed by ER-positive breast cancer cells and CK18 positive cells still underwent estrogen-stimulated cell proliferation suggested that ER-α36-mediated estrogen signaling may stimulate proliferation of breast cancer stem, progenitor and intermediate cells, and also suggested that ER-α36 overexpression might be involved in symmetric stem cell division.

The genomic or classic estrogen-signaling pathway mediated by ER-α66 is prevailingly thought to be responsible for the initiation and progression of breast cancer. However, we found that knock-down of ER-α36 expression in the ER-positive breast cancer cells diminished the tumor-seeding efficiency of the breast cancer stem/progenitor cells and the genomic estrogen-signaling mediated by ER-α66 is attenuated in the ER-positive breast cancer stem/progenitor cells. Additionally, the nuclear expression of ER-α66 is correlated with differentiation of luminal epithelial cells. Our results are in good agreement with a recent report that knock-down of ER-α66 expression in MCF7 cells using the shRNA method was without effect on tumorsphere formation and tumor-seeding potential in nude mice [41]. Together, these results suggested that ER-α36-mediated rapid estrogen signaling plays an important role in maintenance and regulation of ER-positive breast cancer stem/progenitor cells while ER-α66-mediated genomic estrogen signaling is involved in determination of luminal epithelial lineage specific differentiation.

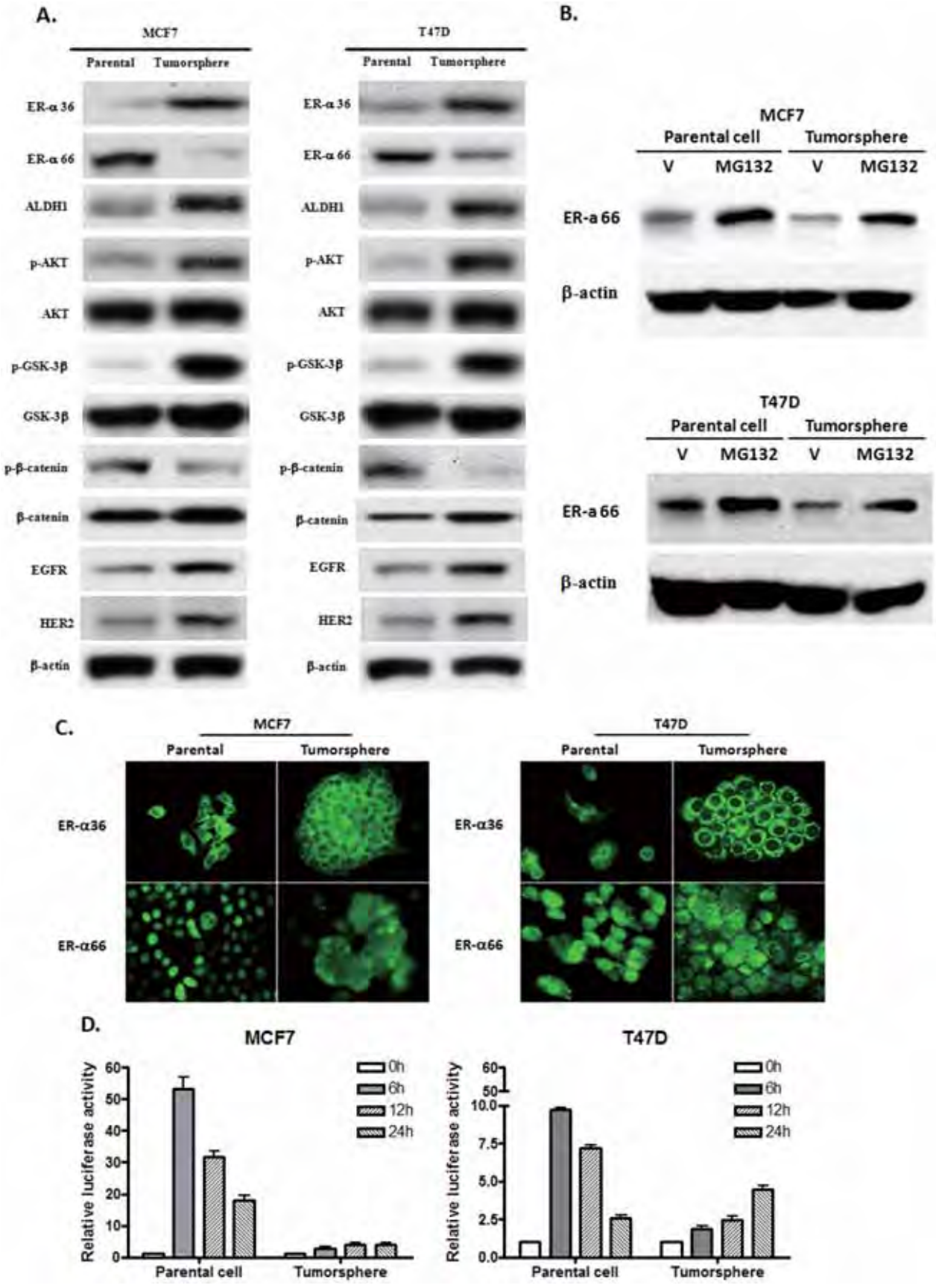
Recently, we reported that ER-positive breast cancer cells expressing high levels of ER-α36 are more resistant to antiestrogen tamoxifen [27], consistent with our previous report that the breast cancer patients with tumors expressing high levels of ER-α36 less benefited from tamoxifen therapy compared to those with low levels of ER-α36 expression, and ER-α36 expression is significantly associated with HER2 expression [22], suggesting that increased ER-α36 expression is one of the underlying mechanisms of tamoxifen resistance. Here, we found that ER-α36 is highly expressed in ER-positive breast cancer stem/progenitor cells and plays an important role in positive regulation of these cells. Taken together, our results suggest that ER-positive breast cancer stem/progenitor cells may be resistant to antiestrogen tamoxifen.

In summary, our results provided strong evidence to support an important role of ER-α36-mediated rapid estrogen signaling in maintenance and regulation of ER-positive breast cancer stem/progenitor cells and provided a rational for development of therapeutic approaches to restrict growth of breast cancer stem/progenitor cells by targeting ER-α36.

Materials and Methods

Reagents, and Antibodies

The 17β-estradiol (E2β) was purchased from Sigma Chemical (St Louis, MO). The PI3K inhibitor LY294002 was from Tocris Bioscience (Ellisville, MO). The GSK-3β inhibitor IX, the AKT inhibitor IV, and the proteasome inhibitor MG132 were purchased from Calbiochem (San Diego, CA). The ER-α36 antibody was generated and characterized as described before [19]. The β-actin antibody (1–19), the goat anti-mouse IgG-HRP, the goat anti-rabbit IgG-HRP and the do nkey anti-goat IgG-HRP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The ER-α antibody (ERAb-16) was purchased from NeoMarkers (Fremont, CA). The antibodies for AKT (#9772), p-AKT (Ser473, #9271), GSK-3βclone7, p-GSK-3βY216/, β-Catenin (clone 14) and p-β-Catenin (Ser33/37/Thr41, #9561) were purchased from Cell Signaling Technology (Danvers, MA). The ALDH1 antibody (#61194) was from BD Biosciences (San Jose, CA). PerCP-Cy^{TM}5.5 mouse anti-human CD44 (clone C26) and PE mouse anti-human CD24 (clone ML5) were purchased from BD Pharmingen (San Jose, CA). Anti-rabbit Alexa Fluor 488 antibody (A-11008) and anti-mouse Alexa Fluor 555 antibody (A-31570) were from Invitrogen (Carlsbad, CA).


A.
MCF7
Parental
Tumorsphere
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Parental
Tumorsphere
ER-α 36
ER-α 66
ALDH1
p-AKT
AKT
p-GSK-3β
GSK-3β
p-β-catenin
β-catenin
EGFR
HER2
β-actin
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MCF7
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ER-a 66
β-actin
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ER-a 66
β-actin
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0h
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Parental cell
Tumorsphere

Figure 7. The expression and genomic function of ER-α66 are down-regulated in ER-positive breast cancer stem/progenitor cells. (A). Western blot analysis of the expression of different proteins in the monolayer cells (parental) and tumorspheres of the MCF7 and T47D cells. (B). Western blot analysis of ER-α66 expression in monolayer (parental) and tumorspheres of the MCF7 and T47D cells treated with or without the proteasome inhibitor MG132 (100 nM) for 12 hours. (C). Indirect Immunofluorescent staining for ER-α36 and ER-α66 in the monolayer cells (parental) and tumorspheres of the MCF7 and T47D cells. (D). The monolayer cells (parental) and tumorspheres of the MCF7 and T47D cells were transfected with the ERE luciferase report plasmid (2 μg). Twenty-four hours later, 0.1 nM of E2β was added and incubated for indicated time periods. The luciferase activities were assayed and normalized using a cytomegalovirus-driven Renilla luciferase plasmid. Two replicates were used in each experiment. Columns: means of four independent experiments; bars, SE.
doi:10.1371/journal.pone.0088034.g007

Cell culture, Establishment of stable cell lines, and Growth assay

MCF7 and T47D cells were purchased from ATCC (Manassas, VA). The cells and their derivatives were cultured in Improved Minimal Essential Medium (IMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino-acids, 1% HEPES buffer, 1% antibiotic-antimycotic from Invitrogen (Carlsbad, CA) and 2 mg/ml bovine insulin (Sigma, St. Louis). All cells were maintained at 37°C and 5% CO2 in a humidified incubator.

MCF7 cells with forced expression of recombinant ER-α36 and with knocked-down levels of ER-α36 expression were established and characterized as described before [26,27]. To establish stable cell lines with knocked-down expression of ER-α36 from T47D cells, we constructed an ER-α36 specific shRNA expression vector by cloning the DNA oligonucleotides 5′-GATGCCAATAG-GTACTGAATTGATATCCGTTCAGTACCTATTGGCAT-3′ targeting the sequence in the 3′UTR of ER-α36 gene into the pRNAT-U6.1/Neo expression vector from GenScript Corp. Piscataway, NJ).

Briefly, T47D cells transfected with the empty expression vector and ER-α36 shRNA expression vector were selected with 500 μg/ml G418 for three weeks, and more than 20 individual clones from transfected cells were pooled, examined for ER-α36 expression with Western blot analysis and retained for experiments.

Tumorsphere formation, Self-renewal and Growth assays

To establish tumorspheres, cells were seeded onto Corning Ultra-Low Attachment 6-well plate (Corning Incorporated, CA) at 10,000 cells/ml and cultured seven days in the tumorsphere medium: phenol-red free DMEM/F12 medium (Invitrogen) supplemented with 1× B-27 (Invitrogen), 20 ng/ml epidermal growth factor (Sigma-Aldrich) and 20 ng/ml basic fibroblast growth factor (ProSpec, NJ), 0.5 μg/mL hydrocortisone (Sigma). Tumorspheres were collected, washed with PBS, and incubated with Trypsin-EDTA (0.25%/0.5 mM) for two minutes at 37°C to dissociated cells, and cells were counted using the ADAM automatic cell counter (Digital Bio, Korea).

To assess the self-renewal of the stem-like cells, tumorspheres were dissociated and cell number was determined. The cells from 1st generation of tumorspheres were seeded onto Ultra-Low Attachment 6-well plate at 5,000 cells/ml and cultured seven days in the tumorsphere medium to form 2nd generation tumorspheres. The cells were then passed once a week for 3rd and 4th generation tumorspheres. The number of tumorspheres and dissociated cells were counted using a Multisizer 3 Coulter Counter (Beckman Coulter, Brea, CA) and the ADAM automatic cell counter, respectively. For estrogen stimulation assays, tumorspheres were treated with 0.1 nM E2β or vehicle (ethanol) as a control. Three dishes were used for each group and all experiments were repeated three times.

Flow Cytometry Analysis

For $CD44^+/CD24^-$ cell analysis, single cell suspension washed with cold PBS/1% BSA were incubated with PerCP-Cy™5.5 mouse anti-human CD44 and PE mouse anti-human CD24 in PBS/1% BSA for 30 minutes at 4°C. After incubation, the cells were washed twice in cold PBS/1% BSA and re-suspended in cold PBS/1% BSA for flow cytometry analysis.

DNA Transfection and Luciferase Assay

T47D and MCF7 cells were transfected with a p2×ERE-Luc reporter plasmid (a kind gift from Dr. Katarine Pettersson at Karolinska Institute, Sweden) using FuGene 6 transfection reagent (Roche Applied Science, Indianapolis, IN). Tumorspheres were transfected with electroporation using a pipette-type electroporator (MicroPorator MP-100, Digital Bio., Korean) as the manufacture recommended. All transfection included a cytomegalovirus-driven Renilla luciferase plasmid, pRL-CMV (Promega, Madison, WI) to establish transfection efficiency. Twenty-four hours after transfection, cells were treated with vehicle or 0.1 nM of E2β for 6, 12 and 24 hours. Cell extracts were prepared and luciferase activities were determined and normalized using the Dual-Luciferase Assay System (Promega, Madison, WI).

Table 1. The relationship between ER-α36, ER-α66 and ALDH1 in sixty-eight patients.

		ER-α36				ER-α66			
		+	−	χ^2	P	+	−	χ^2	P
ER-α66	+	19	13	2.13	>0.05	-	-	-	-
	−	15	21			-	-	-	-
ALDH1	+	20	10	5.96	<0.01	13	17	0.3	>0.05
	−	14	24			19	19		

The immunohistochemistry (IHC) assay was performed in specimens from sixty-eight patients. The results showed that ER-α36 had a positive correlation with ALDH1 ($P<0.01$, $\chi^2 5.96$). There were no correlations between ER-α36 and ER-α66, and between ER-α66 and ALDH1.
doi:10.1371/journal.pone.0088034.t001

Western Blot Analysis

Cells were washed with cold PBS and lysed with the RIPA buffer containing 1% proteinase inhibitor cocktail solution and 1% phosphatase inhibitor cocktail solution (Sigma). The cell lysates were boiled for 5 minutes in sodium dodecyl sulfate (SDS) gel-loading buffer and separated on 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). The membranes were probed with appropriate primary antibodies and visualized with the corresponding secondary antibodies and the ECL kit (Thermo Scientific, Rockford, IL).

Indirect Immunofluorescence Assay

Cells were fixed in 4% paraformaldehyde for 10 minutes, then permeabilized in 0.1% Triton X-100 for 5 minutes, blocked in 1% BSA for 30 minutes, and then incubated with primary antibodies at 4°C overnight. Secondary antibodies, anti-rabbit Alexa Fluor 488 or anti-mouse Alexa Fluor 555 were then added and incubated for 1 hour at room temperature. Cells were washed with PBS and mounted with 10 mg/ml DAPI (4,6-diamidino-2-phenylindole dihydrochloride) (Sigma-Aldrich) in aqueous mountant (Dako, Carpinteria, CA) and photographed using a fluorescent microscope (Nikon, Eclipss E600).

Tumor Seeding Assays in Nude Mice

All animal procedures were approved by the Animal Care and Use Committee at the Creighton University and were performed in compliance with National Institutes of Health guidelines on the ethical use of animals. To assess tumor-seeding efficiency, cells in a serial dilution (1×10^2, 1×10^3, 1×10^4 and 1×10^5 cells) were re-suspended in 0.1 ml of Matrigel and inoculated subcutaneously into the mammary fatpad of ovariectomized female nude mice (5–6 weeks old, strain CDI nu/nu, Charles River Breeding Laboratory). The mice were implanted with 0.35 mg/60-day slow-release 17β-estradiol pellets or placebos (Innovative Research of American, Sarasota, Florida) as controls. Mice were monitored twice a week for tumor growth. At the end of the experiments, the mice were euthanized, and the tumors were removed and weighed.

Statistical Analysis

Data were summarized as the mean ± standard deviation (S.D.) using GraphPad InStat software program. Statistical analysis was performed using paired-samples t-test, or ANOVA followed by the Student–Newman–Keuls testing and the significance was accepted for P values less than 0.05.

Supporting Information

Figure S1 Estrogen failed to influence differentiation of ER-positive breast cancer stem cells cultured on collagen-coated coverslips. The putative stem cells from tumorspheres derived from variants of ER-positive breast cancer MCF7 (A) and T47D (B) cells were cultured on collagen-coated coverslips for five days in the presence of vehicle or 0.1 nM E2β. Indirect Immunofluorescent staining for CD10 (red) and CK18 (red) in the cells. DAPI (blue) indicates the cell nuclei.
(TIF)

Figure S2 Nuclear ER-α66 expression is correlated to CK18 expression in tumorspheres from MCF7 cells. (A). Indirect Immunofluorescent staining for ER-α66 (green) and CK18 (red) in the tumorspheres of the MCF7 cells cultured for three and seven days. DAPI (blue) indicates the cell nuclei.
(TIF)

Figure S3 Immunohistochemical staining of ALDH1, ER-α36 and ER-α66 in a breast cancer specimen. Tissue from one patient showing strong, cytoplasmic and membrane expression of ALDH1 (A) and ER-α36 (B) but no ER-α66 expression (C) (all at ×400 magnification).
(TIF)

Table S1 Summary of tumor formation assay. The ovariectomized female nude mice (5–6 weeks old, strain CDI nu/nu) were implanted with 0.35 mg/60-day slow-release 17β-estradiol pellets or placebos as controls five days before tumor cell injection; n = six mice per group. Tumor cells as indicated in a serial dilution (1×10^2, 1×10^3, 1×10^4 and 1×10^5) were re-suspended in 100 µl of Matrigel and inoculated subcutaneously into the mammary fatpads of nude mice (one tumor per mouse). Tumors from MCF7 variants were harvested at 42 days and T47D variants at 40 days.
(DOCX)

Author Contributions

Conceived and designed the experiments: HD ZYW. Performed the experiments: HD XTZ HYZ . Analyzed the data: HD XTZ MLW HYZ LJL ZYW. Contributed reagents/materials/analysis tools: HD XTZ HYZ ZYW. Wrote the paper: ZYW.

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Disruption of the ER-α36-EGFR/HER2 Positive Regulatory Loops Restores Tamoxifen Sensitivity in Tamoxifen Resistance Breast Cancer Cells

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Abstract

Tamoxifen provided a successful treatment for ER-positive breast cancer for many years. However, most breast tumors develop tamoxifen resistance and are eventually refractory to tamoxifen therapy. The molecular mechanisms underlying development of tamoxifen resistance have not been well established. Recently, we reported that breast cancer cells with high levels of ER-α36, a variant of ER-α, were resistant to tamoxifen and knockdown of ER-α36 expression in tamoxifen resistant cells with the shRNA method restored tamoxifen sensitivity, indicating that gained ER-α36 expression is one of the underlying mechanisms of tamoxifen resistance. Here, we found that tamoxifen induced expression of ER-α36-EGFR/HER2 positive regulatory loops and tamoxifen resistant MCF7 cells (MCF7/TAM) expressed enhanced levels of the loops. Disruption of the ER-α36-EGFR/HER2 positive regulatory loops with the dual tyrosine kinase inhibitor Lapatinib or ER-α36 down-regulator Broussoflavonol B in tamoxifen resistant MCF7 cells restored tamoxifen sensitivity. In addition, we also found both Lapatinib and Broussoflavonol B increased the growth inhibitory activity of tamoxifen in tumorsphere cells derived from MCF7/TAM cells. Our results thus demonstrated that elevated expression of the ER-α36-EGFR/HER2 loops is one of the mechanisms by which ER-positive breast cancer cells escape tamoxifen therapy. Our results thus provided a rational to develop novel therapeutic approaches for tamoxifen resistant patients by targeting the ER-α36-EGFR/HER2 loops.

Citation: Yin L, Zhang X-T, Bian X-W, Guo Y-M, Wang Z-Y (2014) Disruption of the ER-α36-EGFR/HER2 Positive Regulatory Loops Restores Tamoxifen Sensitivity in Tamoxifen Resistance Breast Cancer Cells. PLoS ONE 9(9): e107369. doi:10.1371/journal.pone.0107369

Editor: Ming Tan, University of South Alabama, United States of America

Received May 28, 2014; **Accepted** August 12, 2014; **Published** September 9, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are included within the paper.

Funding: This work was supported by Department of Defense grant DAMD 11-1-0497 and Nebraska Tobacco Settlement Biomedical Research Program Awards (LB-595) to Z.Y. Wang. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Endocrine therapy using antiestrogen tamoxifen (TAM) is currently the most effective treatment for advanced ER-positive breast cancer. Tamoxifen acts through ER pathway, which has been proven to reduce relapse, death rates and risk of contralateral breast cancer. However, patients often develop resistance to tamoxifen, which limit its effectiveness [1–4]. Many researches were conducted to understand the molecular pathways involved in tamoxifen resistance and have revealed that multiple signaling molecules and pathways such as EGFR and HER2 [5,6]. All these pathways often bypass the requirement of estrogen signaling for growth of ER-positive breast cancer cells.

Both experimental and clinical evidence have indicated that the HER2 (Human epidermal growth factor receptor 2) and EGFR (Epidermal growth factor receptor) signaling pathways interact with the estrogen-signaling pathway. Experimental evidence has shown that estrogen-dependent MCF7 cells that over-express HER2 are rendered tamoxifen resistant [5,6]. Hence the HER2 pathway has been investigated for its contribution towards development of tamoxifen resistance and now HER2 has been proposed as a potential marker of tamoxifen sensitivity. Many clinical studies have found an association between HER2 overexpression and tamoxifen failure [7–15]. Thus, the combination therapy by targeting both HER2 and ER-α was hypothesized and tested in preclinical studies [16–18]. Chu et al., reported that the dual kinase inhibitor Laptinib for HER2 and EGFR cooperates with tamoxifen to inhibit cell proliferation in antiestrogen resistant breast cancer [19].

Previously, our laboratory identified and cloned a variant of ER-α, ER-α36, which has a molecular weight of 36-kDa [20,21]. The transcript of ER-α36 is initiated from a previously unidentified promoter in the first intron of the ER-α gene [22]. This ER-α differs from the original 66 kDa ER-α (ER-α66) because it lacks both transcriptional activation domains (AF-1 and AF-2) but retains the DNA-binding and dimerization domains, and partial ligand-binding domain [20]. ER-α36 is mainly expressed at the plasma membrane and mediates membrane-initiated estrogen signaling [21]. Previously, We reported that the breast cancer patients with tumors expressing high levels of ER-α36 less benefited from TAM therapy than those with low levels of ER-α36 expression and ER-α36 expression is well correlated with HER2 expression [23], suggesting that gained ER-α36/HER2 expression is one of the underlying mechanisms of TAM resistance. Indeed, ER-α36 is able to mediate agonist activity of TAM such as activation of the MAPK (mitogen-activated protein

kinase)/ERK (extracellular regulated protein kinases) and the PI3K (Phosphoinositides 3-kinase)/AKT signaling pathways [24,25] and is involved in development of TAM resistance [26,27]. Recently, we reported the existence of positive regulatory loops between ER-α36 and EGFR/HER2 in ER-negative breast cancer cells [28,29]. In triple-negative breast cancer MDA-MB-231 and MDA-MB-436 cells, knockdown of ER-α36 expression enhances EGFR protein degradation through the proteasome system while EGFR signaling pathway up-regulates the promoter activity of ER-α36 through an Ap1 binding site in the 5′ flanking sequence of ER-α36 gene [28]. In HER2 overexpressing breast cancer SKBR3 cells, ER-α36-mediated signaling positively regulates HER2 transcription while HER2 signaling up-regulates the promoter activity of ER-α36. However, the function and underlying mechanisms of these regulatory loops in development of TAM resistance of ER-positive breast cancer cells are largely unknown,

Here, we sought to examine whether the ER-α36-EGFR/HER2 positive regulatory loops also exist in ER-positive breast cancer cells and whether these loops are involved in development of tamoxifen resistance. We also to tested the possibility of disruption of these loops with chemicals to restore TAM sensitivity in TAM resistant cells. Using TAM sensitive breast cancer MCF7 cells and TAM resistant MCF7 cells as models, we investigated the function of the ER-α36-EGFR/HER2 positive regulatory loops in TAM resistance.

Methods

Chemicals and antibodies

Tamoxifen was purchased from Sigma Chemical Co. (St. Louis, MO). Broussoflavonol B was obtained from Shenogen Pharma Group (Beijing, P.R. China). Anti-phospho-EGFR (Tyr1045) and –HER2/ErbB2 (Tyr1221/1222) as well as anti-EGFR and -HER2/ErbB2 (D8F12) antibodies were purchased from Cell Signaling Technology (Boston, MA). Antibodies of ER-α66 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-ER-α36 antibody was generated and characterized as described before [26].

Cell culture and establishment of stable cell lines

MCF7 and T47D cells were obtained from ATCC (American Type Culture Collection, Manassas, VA). H3396 cells were kindly provided by Dr. Leia Smith at the Seattle Genetics Inc. MCF7/ER36, MCF7/Si36, MCF7/TAM and MCF7/TAM/Si36 cells were established as described before [28,30]. All cells were maintained at 37°C in a humidified atmosphere containing 10% CO2 in IMEM media without phenol red and 10% fetal calf serum.

To examine cell growth in the presence or absence of TAM as well as other chemicals, cells maintained for three days in phenol red-free DMEM plus 2.5% dextran-charcoal-stripped fetal calf serum (HyClone, Logan, UT) were treated with different concentrations of TAM and other chemicals, or vehicle as a control. The cells were seeded at 1×10^4 cells per dish in 60 mm dishes and the cell numbers were determined using the ADAM automatic cell counter (Digital Bio., Korea) after seven days. Five dishes were used for each treatment and experiments were repeated more than three times.

Western blot analysis

For immunoblot analysis, cells washed with PBS were lysed with the lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.25 mM EDTA pH 8.0, 0.1% SDS, 1% Triton X-100, 50 mM NaF) supplemented with protease and phosphatase inhibitors (Sigma). The protein amounts were measured using the DC protein assay kit (BIO-RAD Laboratories, Hercules, CA). The same amounts of the cell lysates were boiled for five minutes in loading buffer and separated on a SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane. The membranes were probed with various primary antibodies, HRP-conjugated secondary antibodies, and visualized with enhanced chemiluminescence (ECL) detection reagents (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). All Western blot assays were repeated three times.

Tumorsphere formation and flow cytometry analysis

To establish tumorspheres, cells were seeded onto Corning Ultra-Low Attachment 6-well plate (Corning Incorporated, CA) at 10,000 cells/ml and cultured seven days in the tumorsphere medium: phenol-red free DMEM/F12 medium (Invitrogen) supplemented with 1× B-27 (Invitrogen), 20 ng/ml epidermal growth factor (Sigma-Aldrich) and 20 ng/ml basic fibroblast growth factor (ProSpec, NJ), 0.5 μg/mL hydrocortisone (Sigma). Tumorspheres were collected, washed with PBS, and incubated with Trypsin-EDTA (0.25%/0.5 mM) for two minutes at 37°C to dissociated cells. The number of tumorspheres and dissociated cells were counted using a Multisizer 3 Coulter Counter (Beckman Coulter, Brea, CA) and the ADAM automatic cell counter, respectively. For TAM treatment assays, tumorspheres were treated with tamoxifen or vehicle (ethanol) as a control. Three dishes were used for each group and all experiments were repeated three times.

To assess the effects of disruption of the positive-regulatory loops on the self-renewal of the stem-like cells, tumorspheres were dissociated and cell number was determined. The cells from 1st generation of tumorspheres were seeded onto Ultra-Low Attachment 6-well plate at 5,000 cells/ml and cultured five days in the presence or absence of different chemicals to form 2nd generation tumorspheres. The number of tumorspheres and dissociated cells were counted using a Multisizer 3 Coulter Counter. Three dishes were used for each group and all experiments were repeated three times.

For $CD44^+/CD24^-$ cell analysis, single cell suspension washed with cold PBS/1% BSA were incubated with PerCP-Cy5.5 mouse anti-human CD44 and PE mouse anti-human CD24 in PBS/1% BSA for 30 minutes at 4°C. After incubation, the cells were washed twice in cold PBS/1% BSA and re-suspended in cold PBS/1% BSA for flow cytometry analysis.

Statistical analysis

Data from at least three independent experiments are expressed as the mean ± standard error (SE) using the GraphPad InStat software program. Each data point of cell proliferation and Tumorsphere formation was run at least in triplicates and independent experiments were performed at least three times, Tukey-Kramer Multiple Comparisons Test was also used, and the significance was accepted for $P<0.05$.

Results

Tamoxifen induces ER-α36, EGFR and HER2 expression in ER-positive breast cancer MCF7 cells

Previously, our laboratory identified and cloned a 36 kDa variant of ER-α, ER-α36 that functions differently from the 66 kDa full-length ER-α, ER-α66 [21,22]. Recently, we found that there exist ER-α36-EGFR/HER2 positive regulatory loops in ER-negative breast cancer cells [21. 22]. We then decided to

examine whether the same regulatory loops are also in ER-positive breast cancer cells and involved in development of TAM resistance. The steady state levels of ER-α36, EGFR and HER2 in ER-positive breast cancer MCF7 cells treated with 1 μM of TAM for different time periods were examined with Western blot analysis. After TAM treatment, the levels of ER-α36, EGFR and HER2 expression were dramatically increased in MCF7 cells (Figure 1A), consistent with the previous reports that tamoxifen treatment induced ER-α36 in MCF7 cells [26,27] and EGFR/HER2 expression in MCF7 xenograft tumors [31]. The same results were also observed in ER-positive breast cancer T47D and H3396 cells (Figure 1B & C).

Tamoxifen induces the expression of ER-α36, EGFR and HER2 through the positive regulatory loops in MCF7 cells

To examine if the ER-α36-EGFR/HER2 positive regulatory loops are involved in induction of ER-α36, EGFR and HER2 by TAM, we used a cell line MCF7/Si36; MCF7 cells that express knocked-down levels of ER-α36 [30]. TAM (1 μM) treatment failed to induce ER-α36, EGFR and HER2 expression in this cell line (Figure 2A), indicating that ER-α36 is involved in the induction of EGFR and HER2 by TAM. To confirm this result, we also treated the cells with Broussoflavonol B (BB), an ER-α36 downregulator [32,33] together with TAM. Western blot analysis revealed that BB blocked the induction of ER-α36 as well as EGFR and HER2 by TAM (Figure 2B). Our results thus indicated that ER-α36 is involved in the induction of EGFR and HER2 expression by TAM.

To examine whether the signaling pathways of EGFR and HER2 contribute to the induction of ER-α36 expression by TAM, we treated MCF7 cells with the dual kinase inhibitor Lapatinib together with TAM and found that the EGFR/HER2 dual inhibitor blocked TAM induction of ER-α36 (Figure 2C). Taken together, our results demonstrated that TAM treatment induced expression of ER-α36, EGFR and HER2 presumably through the ER-α36-EGFR/HER2 positive regulatory loops.

Tamoxifen resistant MCF7 cells exhibit enhanced expression of ER-α36-EGFR/HER2 loops

Recently, we reported establishment of a TAM resistant MCF7 cell line (MCF7/TAM) by continuous treatment of TAM sensitive MCF7 cells with 1 μM of TAM for six months [28]. This cell line exhibited resistance to TAM treatment compared to the parental cells and lower concentrations of TAM (≤1 μM) even acted as an agonist in MCF7/TAM cells (Figure 3A). Western blot analysis revealed that ER-α36, EGFR and HER2 were all expressed at higher levels in MCF7/TAM cells compared to the MCF7 parental cells (Figure 3B), suggesting that MCF7 cells gained expression of the ER-α36-EGFR/HER2 regulatory loops during development of acquired TAM resistance.

To confirm that gained expression of ER-α36 is involved in elevated expression of EGFR and HER2, we used a cell line that expressed knocked-down levels of ER-α36 (MCF7/TAM/Si36) [26]. Western blot analysis revealed that both EGFR and HER2 expression was dramatically down-regulated in MCF7/TAM/Si36 cells compared to the control cells transfected with the empty expression vector (Figure 3C). Our data thus suggested that elevated ER-α36 expression is involved in enhanced expression of EGFR and HER2 in TAM resistant breast cancer cells.

To further confirm that enhanced levels of ER-α36 expression contribute to increased expression of both EGFR and HER2, we also used a stable cell line MCF7/ER36 that expresses high levels

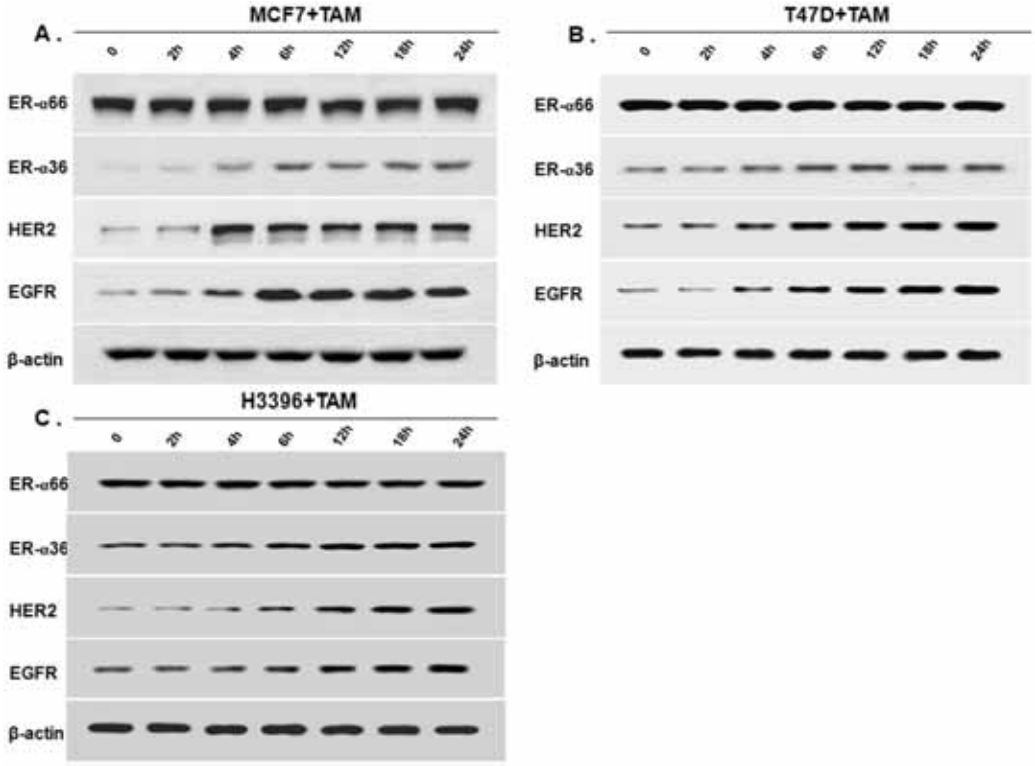

Figure 1. Tamoxifen induces ER-α36, HER2 and EGFR expression. Western blot analysis of the expression levels of ER-α66, ER-α36, HER2 and EGFR in ER-positive breast cancer MCF7 (A), T47D (B) and H3396 (C) cells treated with 1 μM of tamoxifen (TAM) for indicated time period.
doi:10.1371/journal.pone.0107369.g001

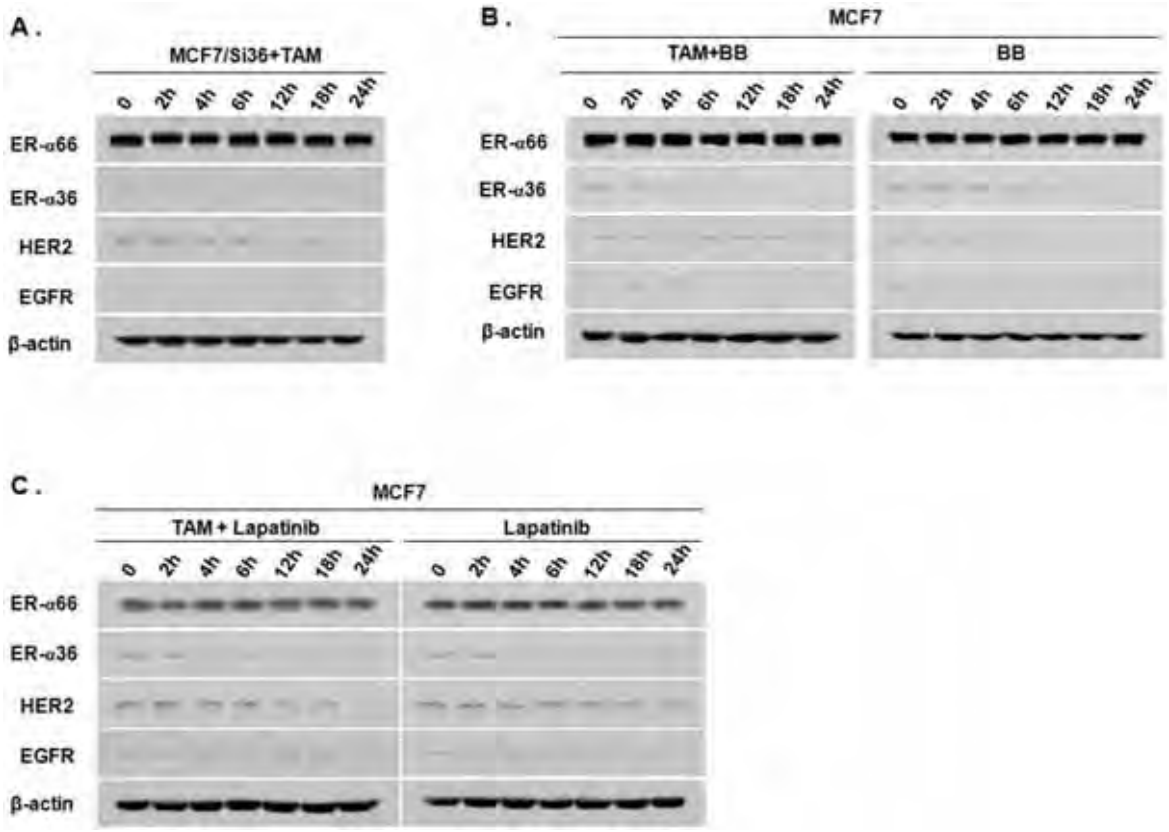

Figure 2. Tamoxifen induces ER-α36, HER2 and EGFR expression via the ER-α36-EGFR/HER2 positive regulatory loops. A. Western blot analysis of the expression levels of ER-α66, ER-α36, HER2 and EGFR in MCF7 cells with knocked-down levels of ER-α36 expression (MCF7/Si36) treated with 1 μM of TAM for indicated time period. B. ER-α66, ER-α36, HER2 and EGFR expression in MCF7 cells treated with 1 μM of TAM together with 1 μM of Broussoflavonol B (BB) for indicated time period. C. Western blot analysis of ER-α66, ER-α36, HER2 and EGFR in MCF7 cells treated with 1 μM of TAM and 1 μM of Lapatinib for indicated time period.
doi:10.1371/journal.pone.0107369.g002

of recombinant ER-α36. Western blot analysis demonstrated that recombinant ER-α36 was highly expressed in MCF7/ER36 cells compared to the control MCF7 cells transfected with the empty expression vector (Figure 3D). Both EGFR and HER2 were also highly expressed in MCF7/ER36 cells (Figure 3D), indicating that increased ER-α36 expression is one of the mechanisms by which ER-positive breast cancer cells gained EGFR and HER2 expression.

Dual kinase inhibitor Lapatinib downregulates ER-α36 expression and sensitizes MCF7/TAM cells to tamoxifen

Previously, we reported that increased level of ER-α36 expression is one of the underlying mechanisms of TAM resistance and knockdown of ER-α36 expression restored TAM sensitivity in MCF7/TAM cells [26]. Here, we sought to examine whether disruption of the ER-α36 and EGFR/HER2 regulatory loops using chemical inhibitors restores TAM sensitivity in TAM resistant MCF7 cells. We first treated MCF7/TAM cells with different concentrations of Lapatinib for 12 hours and the level of ER-α36 expression was examined with Western blot analysis. We found that Lapatinib inhibited phosphorylation of both EGFR and HER2 effectively and also downregulated ER-α36 expression in MCF7/TAM cells (Figure 4A). Lapatinib treatment significantly increased sensitivity to TAM in MCF7/TAM cells (Figure 4B). However, we did not find significant changes of the levels of EGFR and HER2 expression in cells treated with Lapatinib for 12 hours, which seems contradictory to the model of the ER-α36-EGFR/HER2 signaling loops. When we increased Lapatinib treatment to a longer period of time, we observed the expression levels of EGFR and HER2 were dramatically downregulated in the cells treated for 36 hours (Figure 4C). Taken together, these results demonstrated that the dual kinase inhibitor Lapatinib was able to disrupt the ER-α36-EGFR/HER2 positive regulatory loops and restored TAM sensitivity in TAM resistant cells.

ER-α36 downregulator Broussoflavonol B also diminishes EGFR/HER2 expression and restores TAM sensitivity

We then examined whether the ER-α36 downregulator Broussoflavonol B (BB) [32,33] is also able to disrupt the ER-α36-EGFR/HER2 loops and restores TAM sensitivity in TAM-resistant cells. We treated MCF7/TAM cells with different concentrations of BB for 12 hours and the steady state levels of ER-α36, EGFR and HER2 were examined with Western blot analysis. We found that BB potently down-regulated ER-α36 expression and phosphorylation levels of EGFR and HER2 but weakly down-regulated expression levels of EGFR and HER2 proteins in MCF7/TAM cells (Figure 5A). BB treatment also significantly sensitized MCF7/TAM cells to TAM (Figure 5B). When we increased BB treatment to 36 hours, we observed the expression levels of EGFR and HER2 were dramatically downregulated in the cells treated with BB for 36 hours (Figure 4C). Our results thus demonstrated that ER-α36

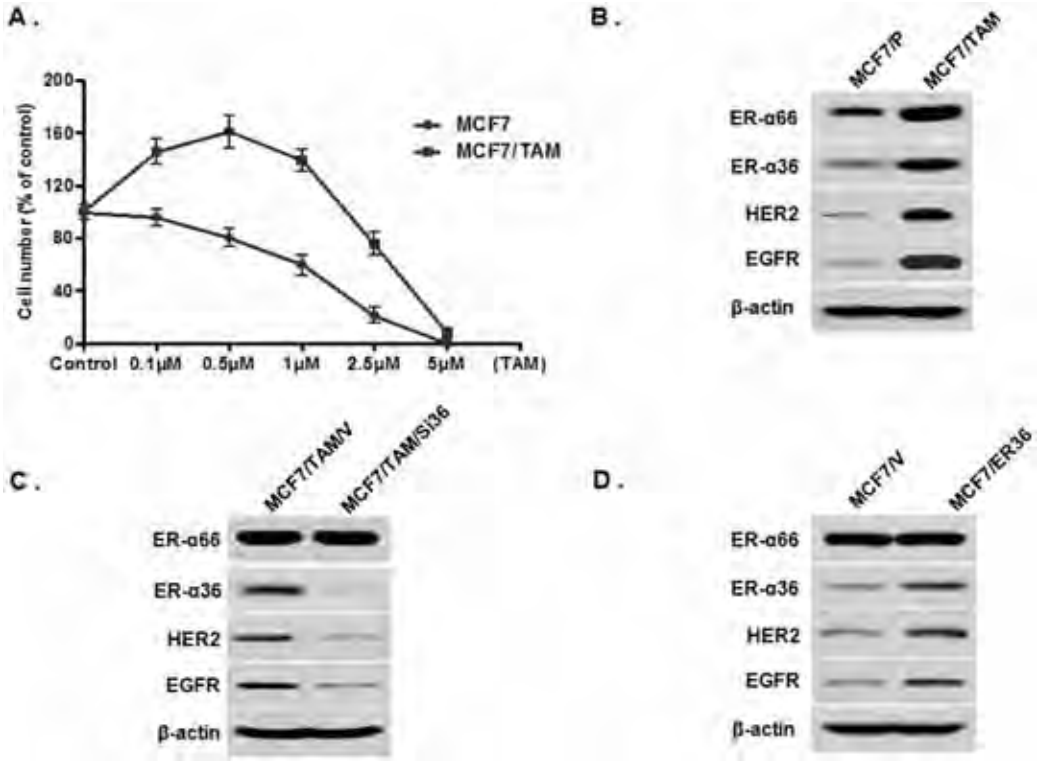

Figure 3. Tamoxifen resistant ER-positive breast cancer MCF7 cells express ER-α36-EGFR/HER2 regulatory loops. A. ER-positive breast cancer MCF7 cells and tamoxifen resistant MCF7 cells (MCF7/TAM) cells were treated with indicated concentrations of TAM for seven days and survived cells were counted. Each point represents the means of three experiments; bars, SE. B. Western blot analysis of the expression levels of ER-α66, ER-α36, EGFR and HER2 in parental MCF7 (MCF7/P) and MCF7/TAM cells. C. Expression of ER-α66, ER-α36, EGFR and HER2 in MCF7/TAM cells transfected with the empty expression vector (MCF7/TAM/V) and MCF7/TAM cells with knocked-down levels of ER-α36 expression (MCF7/TAM/Si36). D. Expression of ER-α66, ER-α36, EGFR and HER2 in MCF7 cells transfected with the empty expression vector (MCF7/V) and MCF7 cells with forced expression of ER-α36 recombinant DNA (MCF7/ER36).
doi:10.1371/journal.pone.0107369.g003

downregulator BB was also able to disrupt the ER-α36-EGFR/HER2 positive regulatory loops and restored TAM sensitivity.

ER-positive breast cancer stem/progenitor cells express ER-α36-EGFR/HER2 positive regulatory loops

Recently, we found that ER-positive breast cancer stem/progenitor cells express higher levels of ER-α36 [33]. We sought to investigate whether there exist ER-α36-EGFR/HER2 regulatory loops in ER-positive breast cancer stem/progenitor cells and disruption of these loops sensitizes these cancer stem/progenitor cells to TAM. We cultured ER-positive breast cancer MCF7 cells in the tumorsphere media and under suspension condition to form tumorspheres, and performed Western blot analysis to assess expression of ER-α36, EGFR and HER2 in tumorsphere and parental cells. We found that ER-α36, EGFR and HER2 were all highly expressed in tumorsphere cells compared to parental cells (Figure 6A). ALDH1, a functional marker of breast cancer stem/progenitor cell [34–36], was also highly expressed in tumorsphere cells (Figure 6A). The results thus demonstrated that there exist ER-α36-EGFR/HER2 regulatory loops in ER-positive breast cancer stem/progenitor cells.

We then sought to examine whether Lapatinib and Broussoflavonol B are still able to disrupt the ER-α36-EGFR/HER2 regulatory loops in ER-positive breast cancer stem/progenitor cells. We cultured MCF7/TAM cells under suspension conditions to form tumorspheres for five days and then Lapatinib (LAP, 5 μM) or Broussoflavonol B (BB, 5 μM) was added for five days. Western blot analysis revealed that both Lapatinib and Broussoflavonol B were able to disrupt the ER-α36-EGFR/HER2 regulatory loops in the breast cancer stem/progenitor cells derived from MCF7/TAM cells (Figure 6B). We also tested the effects of combination of both Lapatinib and Broussoflavonol B on the ER-α36-EGFR/HER2 regulatory loops. We found that the combination treatment also effectively disrupted the ER-α36-EGFR/HER2 regulatory loops (Figure 6C) but failed to observe any synergistic effects of two chemicals.

Disruption of ER-α36-EGFR/HER2 positive regulatory loops sensitizes ER-positive breast cancer stem/progenitor cells to TAM

We then sought to examine whether disruption of the ER-α36-EGFR/HER2 positive regulatory loops will also sensitize ER-positive breast cancer stem/progenitor cells to TAM. We cultured MCF7 cells under suspension conditions to form tumorspheres for five days and then different concentrations of TAM together with Lapatinib (LAP, 5 μM) or Broussoflavonol B (BB, 5 μM) were added for another five days. We found that in the presence of Lapatinib or Broussoflavonol B, ER-positive breast cancer stem/progenitor cells of the tumorspheres formed by MCF7 and MCF7/TAM cells became sensitive to TAM; TAM reduced the number of tumorspheres (Figure 7A & B). We also dissociated cells from tumorspheres and examined cell number of tumorspheres,

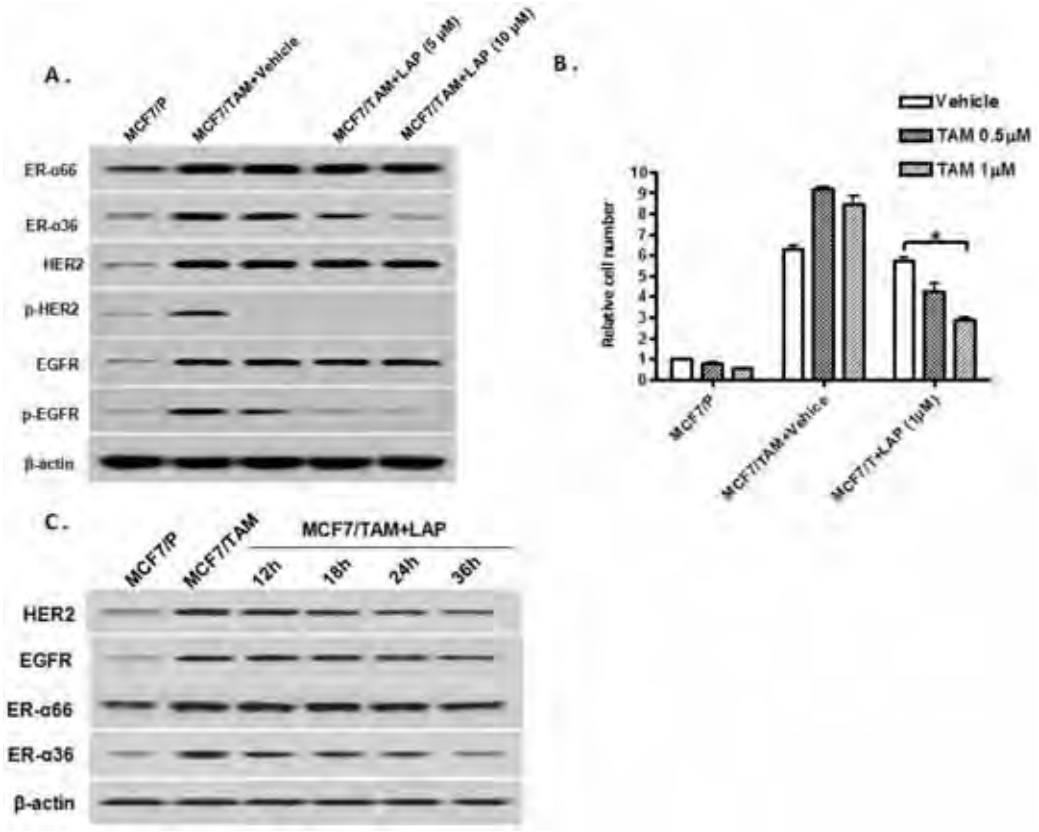

Figure 4. Dual kinase inhibitor Lapatinib downregulates ER-α36 expression and sensitizes MCF7/TAM cells to tamoxifen. A. Western blot analysis of the expression of ER-α36 and 66 as well as EGFR and HER2, and levels of EGFR and HER2 phosphorylation in parental MCF7 (MCF7/P) and MCF7/TAM cells treated with indicated concentrations of Lapatinib for 12 hours. B. Cells were treated with indicated concentrations of TAM together with vehicle or 1 μM of Lapatinib (LAP) for seven days and the numbers of survived cells were counted. The columns represent the means of three experiments; bars, SE. *, $P<0.05$ for MCF/TAM cells treated with vehicle vs cells treated with 1 μM of tamoxifen and Lapatinib.
doi:10.1371/journal.pone.0107369.g004

and found the cell number from the tumorspheres formed by both MCF7 and MCF7/TAM was significantly reduced in the presence of TAM together with Lapatinib or Broussoflavonol B (Figure 7C) while TAM alone enhanced cell growth at lower concentrations in the tumorsphere cells derived from MCF7 and MCF7/TAM cells (Figure 7C).

To assess the effects of disruption of the positive–regulatory loops on the self-renewal of the stem-like cells, we also studied the tumorsphere formation of MCF7 and MCF7/TAM cells as well as their derivatives with different levels of ER-α36 expression through serial passages in the absence or presence of Lapatinib or Broussoflavonol B. The cells were treated with vehicle or chemicals at the time of the sub-seeding. We found that combination of TAM and Lapatinib or Broussoflavonol B dramatically inhibited tumorsphere number in the second generation of tumorspheres (Figure 7 B & C), suggesting that disruption of the positive-regulatory loops also inhibits the self-renewal of the breast cancer stem/progenitor cells. Finally, we tested the effects of combination of both Lapatinib and Broussoflavonol B together with TAM on the growth of stem/progenitor cells derived from MCF7 and MCF7/TAM cells. We found that the combination of two chemicals effectively inhibited tumorsphere formation (Figure 8). Again, we did not observe any synergistic effect.

Discussion

TAM therapy is the most effective treatment for advanced ER-positive breast cancer, but its effectiveness is limited by high rate of resistance acquired during treatment. Previously, we reported that breast cancer patients with tumors expressing high levels of endogenous ER-α36 less benefited from TAM therapy than those with low levels of ER-α36 expression [23], suggesting elevated expression of ER-α36 is a mechanism underlying acquired tamoxifen resistance. Recently, others and we confirmed that elevated ER-α36 expression is involved in TAM resistance through mediating agonist activity of TAM [26,27].

Here, we showed that TAM induced expression of ER-α36, EGFR and HER2 in TAM sensitive MCF7, T47D and H3396 cells. In addition, MCF7/TAM cells selected by long-term cultivation in the presence of TAM also expressed elevated levels of endogenous ER-α36, EGFR and HER2, which results in more rapid cell growth compared to the parental MCF7 cells. Knockdown of ER-α36 expression in MCF7/TAM cells reduced EGFR and HER2 expression. We also showed that MCF7 cells with forced expression of ER-α36 expressed increased levels of EGFR and HER2. Taken together, our results indicated that the ER-α36-EGFR/HER2 positive regulatory loops are one of the underlying mechanisms of ER-positive breast cancer cells gained expression of the growth factor receptors during TAM treatment.

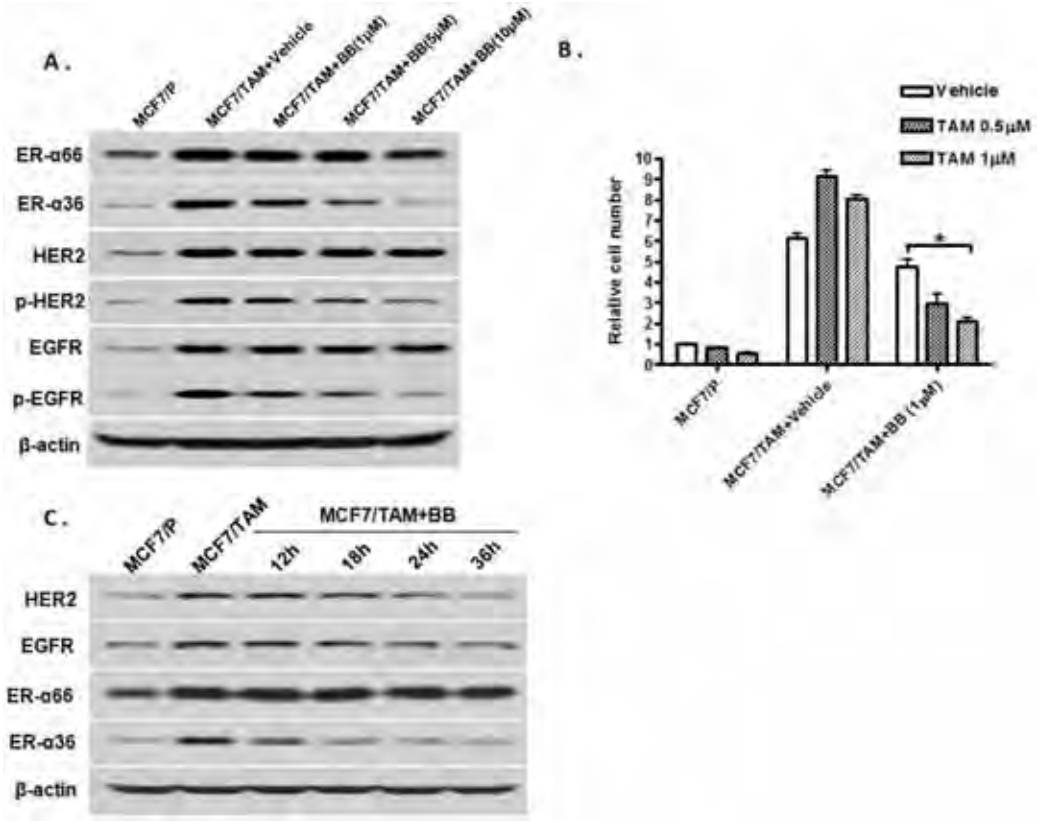

Figure 5. ER-α36 disruptor Broussoflavonol B restores TAM sensitivity. A. Western blot analysis of the expression of ER-α36, ER-α66, EGFR and HER2, and levels of EGFR and HER2 phosphorylation in parental MCF7 (MCF7/P) and MCF7/TAM cells treated with indicated concentrations of Broussoflavonol B (BB) for 12 hours. B. Cells were treated with indicated concentrations of tamoxifen (TAM) together with vehicle or 1 μM of Broussoflavonol B (BB) for seven days and the numbers of survived cells were counted. The columns represent the means of three experiments; bars, SE. *, $P<0.05$ for MCF/TAM cells treated with vehicle vs cells treated with 1 μM of tamoxifen and BB.
doi:10.1371/journal.pone.0107369.g005

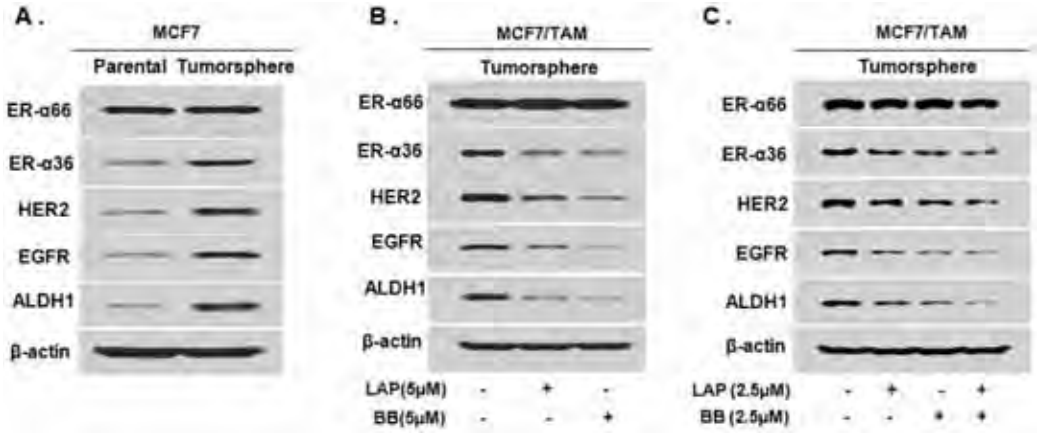

Figure 6. Tumorsphere cells derived from ER-positive breast cancer MCF7 cells express ER-α36-EGFR/HER2 positive regulatory loops. A. Western blot analysis of the expression of ER-α36, ER-α66, EGFR, HER2 and ALDH1 in the monolayer MCF7 cells grown on attachment dishes (MCF7/P) and MCF7 tumorsphere cells grown on low-attachment dishes. B. Tumorsphere cells derived from MCF7TAM cells were treated with 5 μM of Broussoflavonol B (BB) or Lapatinib (LAP) for five days. Western blot analysis of expression levels of different proteins was performed. C. Tumorsphere cells derived from MCF7TAM cells were treated with 2.5 μM of Broussoflavonol B (BB), Lapatinib (LAP) or BB (2.5 μM) and LAP (2.5 μM) together for five days. Western blot analysis of expression levels of different proteins was performed.
doi:10.1371/journal.pone.0107369.g006

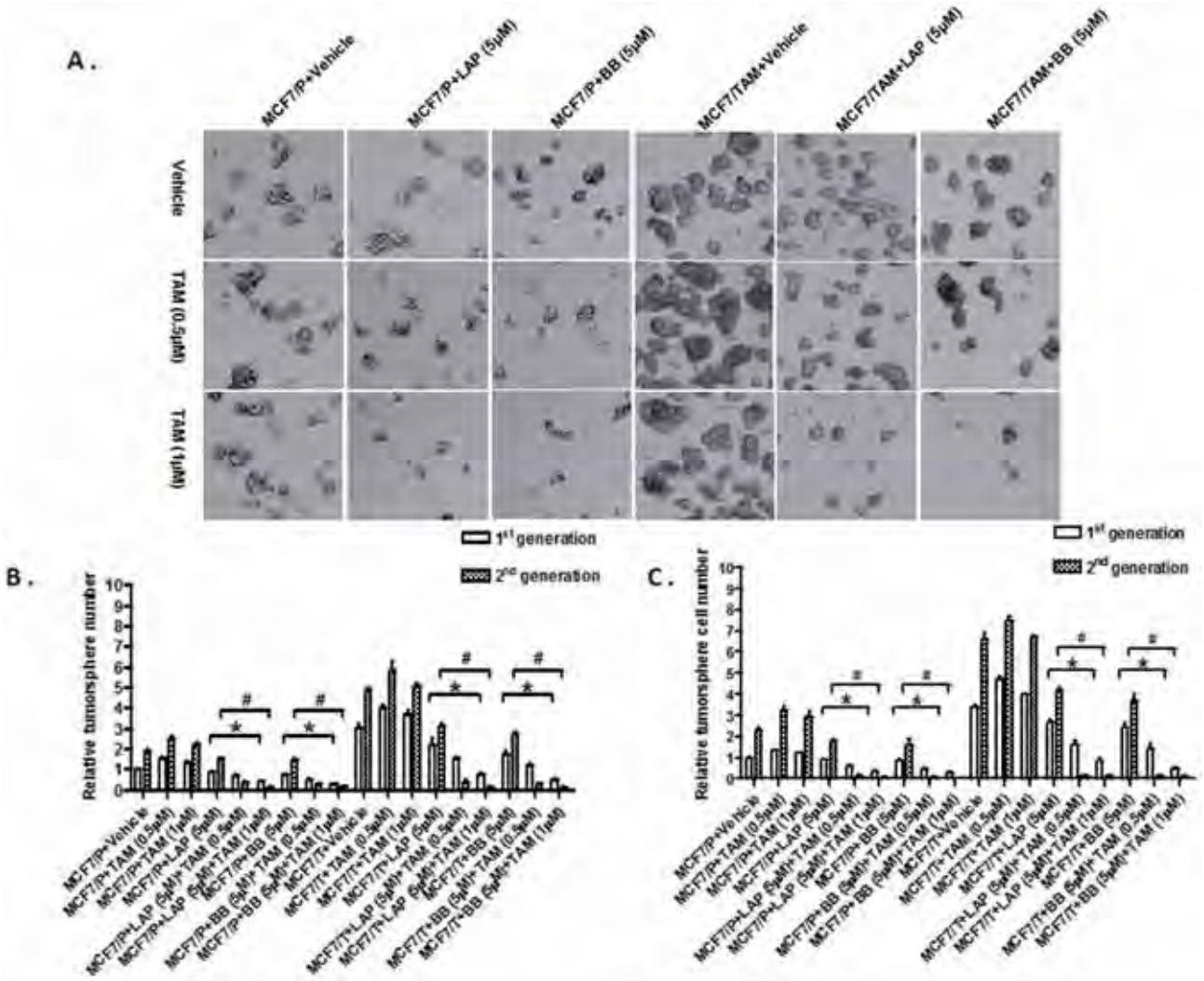

Figure 7. Disruption of the ER-α36-EGFR/HER2 positive regulatory loops sensitizes ER-positive breast cancer stem/progenitor cells to TAM. A. Tumorsphere formation assay was used to assess the effects of TAM alone or together with Lapatinib (LAP) or Broussoflavonol B (BB) on ER-positive breast cancer stem/progenitor cells derived from parental MCF7 (MCF7/P) and tamoxifen resistant MCF7 cells (MCF7/TAM). The representative results are shown. B. The numbers of tumorspheres formed by MCF7/P and MCF7/TAM cells in the presence or absence of LAP and BB for 1st and 2nd generations. C. The number of cells from dissociated tumorspheres formed by MCF7/P and MCF7/TAM cells in the presence or absence of LAP and BB for 1st and 2nd generations. The columns represent the means of three experiments; bars, SE. * & #, $P<0.05$ for MCF/TAM cells treated with vehicle vs cells treated with tamoxifen and LAP or BB, respectively.
doi:10.1371/journal.pone.0107369.g007

Previously, we found that antiestrogens TAM and ICI 182, 780 failed to block ER-α36-mediated non-genomic estrogen signaling [21]. Recently, we also found that ER-α36 mediated agonist activities of both TAM and ICI 182, 780 in ER-negative breast cancer cells that express high levels of endogenous ER-α36 [24] and elevated ER-α36 expression is one of the underlying mechanisms of TAM resistance [26,27]. Here we showed that MCF7/TAM cells exhibited a biphasic growth curve in response to TAM; increasing cell growth at low concentrations and failed to do so at higher concentrations, consistent with previous findings that some breast cancers may be initially growth inhibited by TAM, and later become dependent on TAM for proliferation [5,6]. It worth noting that the TAM at higher concentrations was still able to inhibit growth of MCF7/TAM cells, suggesting that TAM resistance is a concentration dependent event; high concentrations of TAM still exhibit cytotoxic activity in cells insensitive to low concentrations of TAM.

In the current study, we observed that TAM induced ER-α36, EGFR and HER2 expression through the positive regulatory loops and inhibition of both EGFR and HER2 signaling pathways with the dual kinase inhibitor Lapatinib disrupted the regulatory loops and restored TAM sensitivity. Our results thus are in good agreement with the previous reports that Lapatinib restores antiestrogen sensitivity in breast cancer cells with acquired endocrine resistance [19,37]. Here, we observed that Lapatinib inhibited phosphorylation of both EGFR and HER2 and downregulated ER-α36. Our data thus provided a novel molecular mechanism to the function of the dual kinase inhibitor Lapatinib; disruption of the ER-α36-EGFR/HER2 positive regulatory loops. It is worth noting that it has been reported that Lapatinib at 1 μM modestly induced HER2 expression in ER-negative breast cancer

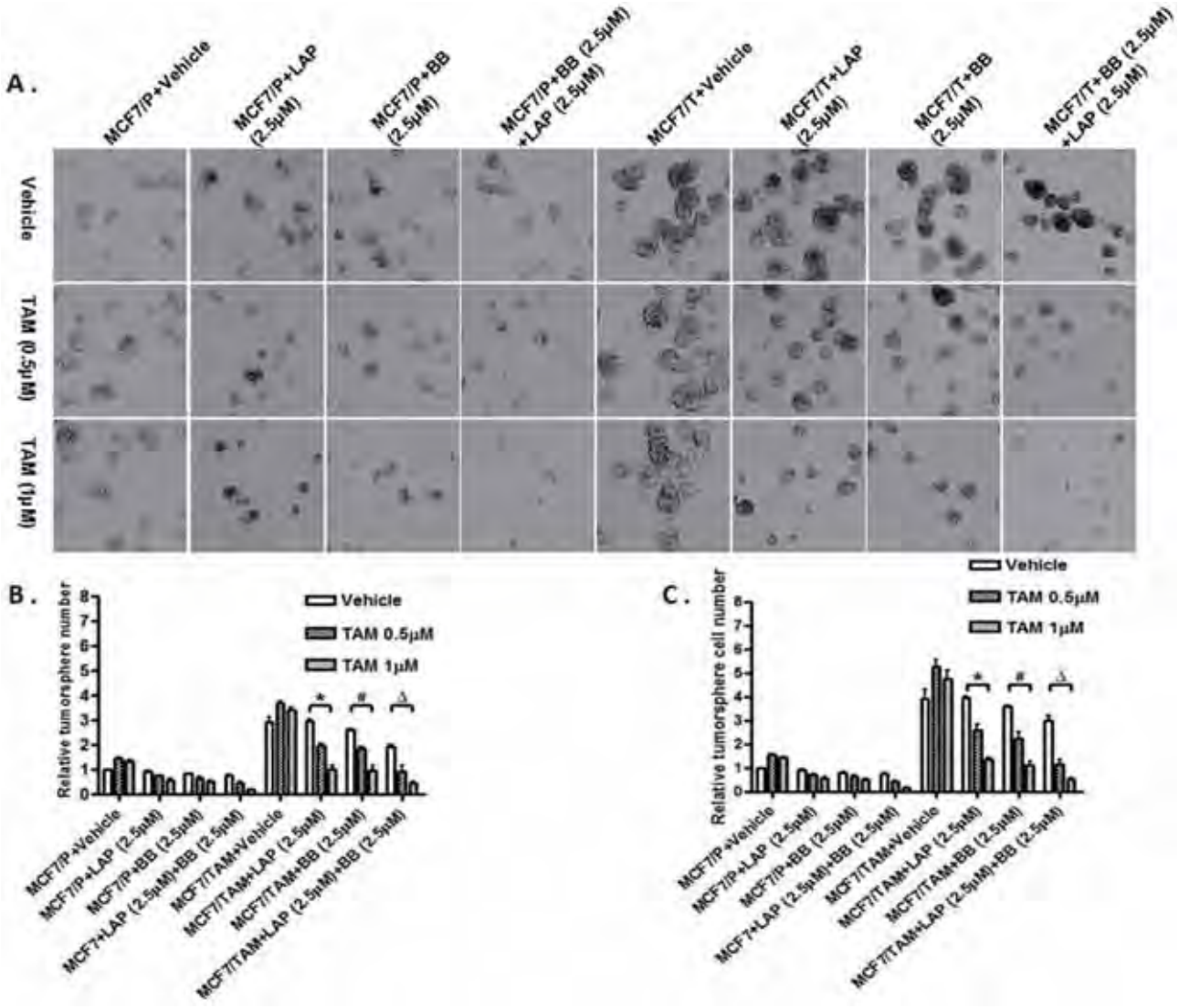

Figure 8. Combination of Broussoflavonol B and Lapatinib sensitizes ER-positive breast cancer stem/progenitor cells to TAM. A. Tumorsphere formation assay was used to assess the effects of TAM alone or together with 2.5 μM of Lapatinib (LAP), Broussoflavonol B (BB) or BB (2.5 μM) and LAP (2.5 μM) on ER-positive breast cancer stem/progenitor cells derived from parental MCF7 (MCF7/P) and tamoxifen resistant MCF7 cells (MCF7/TAM). The representative results are shown. B. The numbers of tumorspheres formed by MCF7/P and MCF7/TAM cells in the absence or presence of LAP (2.5 μM), BB (2.5 μM) or LAP (2.5 μM) and BB (2.5 μM) together. C. The number of cells from dissociated tumorspheres formed by MCF7/P and MCF7/TAM cells in the absence or presence of LAP (2.5 μM), BB (2.5 μM) or LAP (2.5 μM) and BB (2.5 μM) together. The columns represent the means of three experiments; bars, SE. *, # and Δ, $P<0.05$ for MCF/TAM cells treated with vehicle vs cells treated with tamoxifen and LAP, BB, or LAP+BB together.
doi:10.1371/journal.pone.0107369.g008

SKBR3 and MCF7-HER2 cells that over-express HER2 [38], which seems contradictory to our findings. We performed experiments with different concentrations of Lapatinib in MCF7/HER2-18 cells that were stably transfected with a HER2 expression vector [39] and found that Lapatinib at 100 nM and 1 μM indeed modestly increased HER2 expression while higher concentrations of Lapatinib (>1 μM) decreased the steady state level of HER2 (data not shown). Thus, Lapatinib regulation of HER2 expression may be a concentration dependent event.

Previously, we found that the potent ER-α disruptor ICI 182, 780 failed to degrade ER-α36 due to the lacking of the critical Helix 12 in the C-terminal of ER-α36 protein [40]. Recently, we found that a falconoid, Broussoflavonol B (5, 7, 3', 4'-Tetrahydroxy-3-methoxy-6,8-diprenylflavone) purified from the bark of Broussonetia papyrifera was able to downregulate ER-α36 expression and to inhibit proliferation of ER-negative breast cancer cells [32,33]. Here, we showed that Broussoflavonol B was also able to disrupt the ER-α36-EGFR/HER2 positive regulatory loops and restored TAM sensitivity in TAM resistant cells. We also showed that Broussoflavonol B treatment was able to block the induction of the positive regulatory loops by TAM in MCF7 cells. Thus, further development of chemical compounds like Broussoflavonol B may provide novel approaches to restore TAM sensitivity in TAM resistant cells or to block the development of acquired TAM resistance.

Accumulating experimental and clinical evidence indicate that breast cancer arises from mammary stem/progenitor cell populations [41–43]. Although the possible involvement of breast cancer stem/progenitor cells in TAM resistance has been proposed [44] and demonstrated [45], the exact function and the underlying mechanism of breast cancer stem/progenitor cells in TAM resistance remain largely unknown. Many signaling pathways involved in regulation of normal mammary stem cells including Hedgehog, Bmi-1, Wnt, NOTCH, HER2, p53 and PTEN/Akt/β-catenin pathways play roles in breast cancer stem/progenitor cells [46–49]. Recently, we reported that ER-positive breast cancer stem/progenitor cells express higher levels of ER-α36 [34]. Here, we showed that stem-like cells in the tumorspheres derived from MCF7 express elevated levels of ER-α36, EGFR and HER2, indicating there exist the ER-α36-EGFR/HER2 regulatory loops in the ER-positive breast cancer stem/progenitor cells. Again, disruption of these regulatory loops with Lapatinib or Broussoflavonol B was able to sensitize ER-positive breast cancer stem/progenitor cells to TAM. Our results thus provided rationales to develop novel therapeutic approaches to treat breast cancer via eliminating breast cancer stem/progenitor cells by targeting the ER-α36-EGFR/HER2 loops.

In this study, we also tested the effects of combination of both Lapatinib and Broussoflavonol B on the ER-α36-EGFR/HER2 regulatory loops and on the growth of tumorsphere cells derived from MCF7/TAM cells. We found that the combinational treatment effectively disrupted the ER-α36-EGFR/HER2 regulatory loops and inhibited growth of tumorsphere cells. However, we did not observe any synergistic effects of two chemicals, which is in good agreement with our hypothesis that both Lapatinib and Broussoflavonol B affect the same ER-α36-EGFR/HER2 regulatory loops.

In summary, here we provided evidence to demonstrate the existence of ER-α36-EGFR/HER2 positive regulatory loops in TAM resistant breast cancer cells and that disruption of these regulatory loops restored TAM sensitivity in these cells. Our findings that elevated expression of the ER-α36-EGFR/HER2 regulatory loops is one of the mechanisms by which ER-positive breast cancer cells escape the hormonal therapy based on estrogen deprivation provided a rational to develop novel therapeutic approaches for TAM resistant patients by targeting these regulatory loops.

Author Contributions

Conceived and designed the experiments: ZYW LY XTZ. Performed the experiments: LY XTZ. Analyzed the data: LY XTZ. Contributed reagents/materials/analysis tools: ZYW. Contributed to the writing of the manuscript: ZYW. Experimental technical support: XWB YMG.

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Journal of Steroid Biochemistry & Molecular Biology 144 (2014) e417–e426

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

ER-α variant ER-α36 mediates antiestrogen resistance in ER-positive breast cancer stem/progenitor cells

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ARTICLE INFO

Article history:
Received 14 March 2014
Received in revised form 29 May 2014
Accepted 21 August 2014
Available online 23 August 2014

Keywords:
ER-α36
Antiestrogen resistance
ER-positive breast cancer
Cancer stem/progenitor cells

ABSTRACT

Accumulating evidence indicates that cancer stem cells (CSC) play important roles in breast cancer occurrence, recurrence and metastasis as well as resistance to therapy. However, the roles of breast cancer stem cells in antiestrogen resistance and the underlying molecular mechanisms have not been well established. Previously, we identified and cloned a novel variant of estrogen receptor α, ER-α36, with a molecular weight of 36 kDa. ER-α36 mediates rapid antiestrogen signaling and is highly expressed in ER-positive breast cancer stem/progenitor cells. In this study, we investigated the function and the underlying mechanism of ER-α36-mediated antiestrogen signaling in ER-positive breast cancer stem/progenitor cells. ER-positive breast cancer cells MCF7 and T47D as well as variants with different levels of ER-α36 expression were used. The effects of antiestrogens tamoxifen and ICI 182, 780 on breast CSC's ability of growth, self-renewal, differentiation and tumor seeding were examined using tumorsphere formation, flow cytometry, indirect immunofluorences and *in vivo* xenograft assays. The underlying mechanisms were also analyzed with Western blot analysis. We found that the cancer stem/progenitor cells enriched from ER-positive breast cancer cells were more resistant to antiestrogens than the bulk cells. Antiestrogens increased the percentages of the stem/progenitor cells from ER-positive breast cancer cell through stimulation of luminal epithelial lineage specific ER-positive breast cancer progenitor cells while failed to do so in the cells with knocked-down levels of ER-α36 expression. Our results thus indicated that ER-α36-mediated antiestrogen signaling such as the PI3K/AKT plays an important role in antiestrogen resistance of ER-positive breast cancer stem/progenitor cells.

1. Introduction

Estrogen profoundly influences breast cancer development, which highlights the importance of antiestrogen therapy. However, despite the significant anti-neoplastic activities of antiestrogens, most breast tumors are eventually resistant to antiestrogen therapy. Many initially responsive breast tumors gradually acquire antiestrogen resistance by loss of antiestrogen responsiveness. The mechanisms by which breast tumors loss their antiestrogen responsiveness have not been well established. Several mechanisms have been postulated to be involved in the antiestrogen resistance such as increased growth factor signaling, altered expression of co-regulators, mutations of ER-α [1–3].

Accumulating experimental and clinical evidence indicate that breast cancer arises from mammary stem/progenitor cell populations [4–6]. The major features of cancer stem cells include the ability of self-renewal and generating tumors from very few cells, slow cell division, the ability to produce differentiated cells of different lineages, selective resistance to radio- and chemo-therapy [7,8], constitutive activation of anti-apoptotic pathways and induction of angiogenesis, the ability to migrate and spread in metastasis [9]. Although the possible involvement of breast cancer stem/progenitor cells in antiestrogen resistance has been proposed [10] and demonstrated [11], the exact function and the underlying mechanism of breast cancer stem/progenitor cells in antiestrogen resistance remain largely unknown.

Previously, we identified and cloned a variant of ER-α that has a molecular weight of 36 kDa and was named ER-α36 [12,13].

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http://dx.doi.org/10.1016/j.jsbmb.2014.08.017
0960-0760/

ER-α36 is mainly located near the plasma membrane and in the cytoplasm, and mediates rapid estrogen signaling such as activation of the MAPK/ERK [13]. Recently, we reported that the well-known "pure" ER disruptor ICI 182, 780 (Fulvestrant or Faslodex) failed to down-regulate ER-α36 expression [14], and ER-α36 mediated agonist activity of antiestrogens tamoxifen and ICI 186, 780 such as activation of the MAPK/ERK and the PI3K/AKT signaling pathways [15–17]. The breast cancer patients with tumors expressing high levels of ER-α36 less benefited from tamoxifen therapy compared to those with low levels of ER-α36 expression, and ER-α36 expression is significantly associated with HER2 expression [18], suggesting that increased ER-α36 expression is one of the underlying mechanisms of tamoxifen resistance.

Recently, we reported that ER-α36 plays an important role in tamoxifen resistance; tamoxifen resistant cells express high levels of ER-α36 and knockdown of ER-α36 expression in these cells restored tamoxifen sensitivity [17]. More recently, we found that ER-α36 is involved in positive regulation and maintenance of ER-positive breast cancer stem/progenitor cells [19].

In the current study, we investigated the function and the underlying mechanisms of ER-positive breast cancer stem/progenitor cells in antiestrogen resistance and found that ER-α36 plays an important role in antiestrogen resistance of ER-positive breast cancer stem/progenitor cells.

2. Materials and methods

2.1. Reagents and antibodies

Tamoxifen was purchased from Sigma Chemical (St. Louis, MO, USA) and the ICI 182, 780 was from Tocris Bioscience (Ellisville, MO, USA). The affinity-purified rabbit polyclonal anti-ER-α36 antibody was generated as a custom service from Pacific Immunology Corp. (Ramona, CA, USA). The antibody was raised against a synthetic peptide antigen corresponding to the unique C-terminal 20 amino acids of ER-α36. The specificity of the antibody was tested in ER-α36 expression vector transfected HEK293 cells that do not express endogenous ER-α36. Immunofluorescence assay was also used to demonstrate immunoreactive signals only in transfectants with the ER-α36-expressing vectors but not in transfectants harboring an empty expression vector (data not shown).

The β-actin antibody (1-19), anti-CK18 (DC-10) and anti-CD10 (H-321) antibodies, anti-PCNA antibody (FL-261), the goat anti-mouse IgG–HRP, the goat anti-rabbit IgG–HRP and the donkey anti-goat IgG–HRP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The ER-α antibody (ERAb-16) was purchased from NeoMarkers (Fremont, CA, USA). The antibodies for AKT and p-AKT (Ser473) were purchased from Cell Signaling Technology (Danvers, MA, USA). The ALDH1 antibody was from BD Biosciences (San Jose, CA). PerCP-Cy™5.5 mouse anti-human CD44 (clone C26) and PE mouse anti-human CD24 (clone ML5) were purchased from BD Pharmingen (San Jose, CA, USA). Anti-rabbit Alexa Fluor 488 antibody and anti-mouse Alexa Fluor 555 antibody were from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture, establishment of stable cell lines, and growth assay

MCF7 and T47D cells were purchased from ATCC (Manassas, VA, USA). The cells and their derivatives were cultured in Improved Minimal Essential Medium (IMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino-acids, 1% HEPES buffer, 1% antibiotic-antimycotic from Invitrogen (Carlsbad, CA, USA) and 2 mg/ml bovine insulin (Sigma, St. Louis, MO, USA). All cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

The variants of MCF7 and T47D with different levels of ER-α36 expression were established as described before [17,19]. For antiestrogen treatment, cells grown on attachment dishes were treated with different concentrations of antiestrogens or vehicle (ethanol) as a control for five to seven days as indicated. Treated cells were counted using the ADAM automatic cell counter (Digital Bio, Korea). Three dishes were used for each treatment and experiments were repeated three times.

2.3. Tumorsphere formation and growth assays

To form tumorspheres, cells were seeded into Corning ultra-low attachment 6-well plate (Corning Incorporated, CA, USA) at 10,000 cells/ml and cultured seven days in the tumorsphere medium: phenol-red free DMEM/F12 medium (Invitrogen) supplemented with 1 X B-27 (Invitrogen), 20 ng/ml epidermal growth factor (Sigma–Aldrich) and 20 ng/ml basic fibroblast growth factor (ProSpec, NJ, USA), 0.5 μg/ml hydrocortisone (Sigma). The number of tumorspheres was counted using a Multisizer 3 Coulter Counter (Beckman Coulter, Brea, CA). In addition, tumorspheres were then collected, washed with PBS, and incubated with Trypsin-EDTA (0.25%, 0.5 mM) for two minutes at 37 °C to dissociated cells, and cells were counted using the ADAM automatic cell counter (Digital Bio, Korea).

For antiestrogen treatment, tumorspheres were treated with different concentrations of antiestrogens or vehicle (ethanol) as a control. Three dishes were used for each treatment and experiments were repeated three times.

2.4. Flow cytometry analysis

For $CD44^+/CD24^-$ cell analysis, single cell suspension washed with cold PBS/1% BSA were subsequently incubated with PerCP-Cy™5.5 mouse anti-human CD44 (1:20) and PE mouse anti-human CD24 (1:5) in PBS/1% BSA for 30 min at 4 °C. After incubation, the cells were washed twice with cold PBS/1% BSA and re-suspended in 400 μl cold PBS/1% BSA for flow cytometry analysis.

2.5. Western blot analysis

Cells were washed with cold PBS and lysed with the RIPA buffer containing 1% proteinase inhibitor cocktail solution and 1% phosphatase inhibitor cocktail solution (Sigma). The cell lysates were boiled for 5 min in sodium dodecyl sulfate (SDS) gel-loading buffer and separated on 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with appropriate primary antibodies and visualized with the corresponding secondary antibodies and the ECL kit (Thermo Scientific, Rockford, IL, USA).

2.6. Indirect immunofluorescence assay

Cells were fixed in 4% paraformaldehyde for 10 min, then permeabilized in 0.1% Triton X-100 for 5 min, blocked in 1% BSA for 30 min, and then incubated with primary antibodies at 4 °C overnight. Secondary antibodies, anti-rabbit Alexa Fluor 488 or anti-mouse Alexa Fluor 555 were then added and incubated for 1 h at room temperature. Cells were washed with PBS and mounted with 10 mg/ml DAPI (4,6-diamidino-2-phenylindole dihydrochloride) (Sigma–Aldrich) in aqueous mountant

(Dako, Carpinteria, CA) and photographed using a fluorescent microscope (Nikon, Eclipss E600).

2.7. Tumor seeding assays in nude mice

All animal procedures were approved by the Animal Care and Use Committee at the Creighton University and were performed in compliance with National Institutes of Health guidelines on the ethical use of animals. To assess tumor-seeding efficiency, cells in a serial dilution (1×10^2 and 1×10^3 cells) were re-suspended in 0.1 ml of Matrigel (BD Biosciences, San Jose, CA) and inoculated subcutaneously into the mammary fatpad of ovariectomized female nude mice (5–6 weeks old, strain CDI nu/nu, Charles River Breeding Laboratory). The mice were implanted with 0.35 mg/60-day slow-release 17β-estradiol pellets or placebos (Innovative Research FL, USA) as controls. Mice were monitored twice a week for tumor growth. At the end of experiment, all mice were sacrificed using carbon dioxide euthanasia, and the tumors were removed and weighed.

2.8. Statistical analysis

Data were summarized as the mean ± standard deviation (S.D.) using GraphPad InStat software program. Statistical analysis was performed using paired-samples *t*-test, or ANOVA followed by the Student–Newman–Keuls testing and the significance was accepted for *P* values less than 0.05.

3. Results

3.1. ER-positive breast cancer stem/progenitor cells are resistant to antiestrogens

Recently, we reported that ER-α36 is involved in tamoxifen resistance of ER-positive breast cancer cells by mediating tamoxifen-induced activation of the PI3K/AKT pathway [17] and ER-α36 plays an important role in maintenance of the ER-positive breast cancer stem/progenitor cells [19]. We decided to examine whether ER-positive breast cancer stem/progenitor cells are resistant to antiestrogens tamoxifen and ICI 182, 780. We cultured ER-positive breast cancer MCF7 and T47D cells in the tumorsphere media and under suspension condition to form tumorspheres, and performed Western blot analysis to assess ER-α36 expression in tumorsphere and parental cells. We found that ER-α36 was highly expressed in tumorsphere cells compared to parental cells while ER-α66 expression was down regulated in tumorsphere cells (Fig. 1A and B). ALDH1, a functional marker of breast cancer stem/progenitor cell [20,21], was also highly expressed in tumorsphere

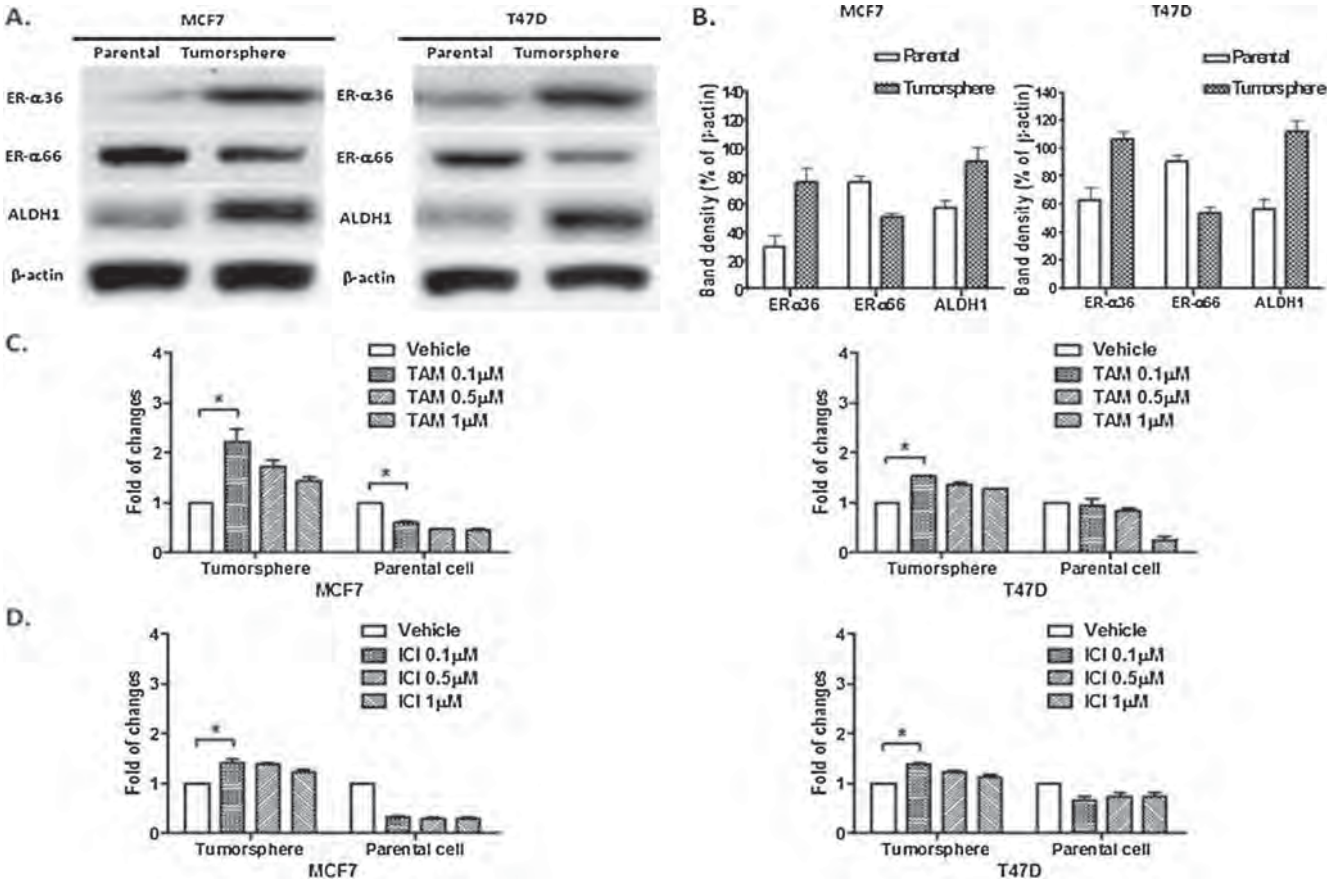

Fig. 1. Resistance of ER-positive breast cancer stem/progenitor cells to antiestrogens. (A). Western blot analysis of ER-α36 and ER-α66, ALDH1 expression in the tumorspheres formed by ER-positive breast cancer MCF7 and T47D cells. (B). The band density relative to α-actin with each column represents the means of three experiments; bars, SE. (C and D). Cells in the tumorspheres formed by MCF7 and T47D cells are more resistant to antiestrogens than parental cells. The tumorspheres cultured in suspension and the monolayer parental cells cultured in attachment dishes were treated with indicated antiestrogens for seven days, and the cell number was determined. The columns represent fold of changes with the cell number treated with vehicle was arbitrarily set as 1. The experiment was repeated three times; bars, SE. *, $P < 0.05$ for cells treated with vehicle vs cells treated with antiestrogens at 0.1 μM.

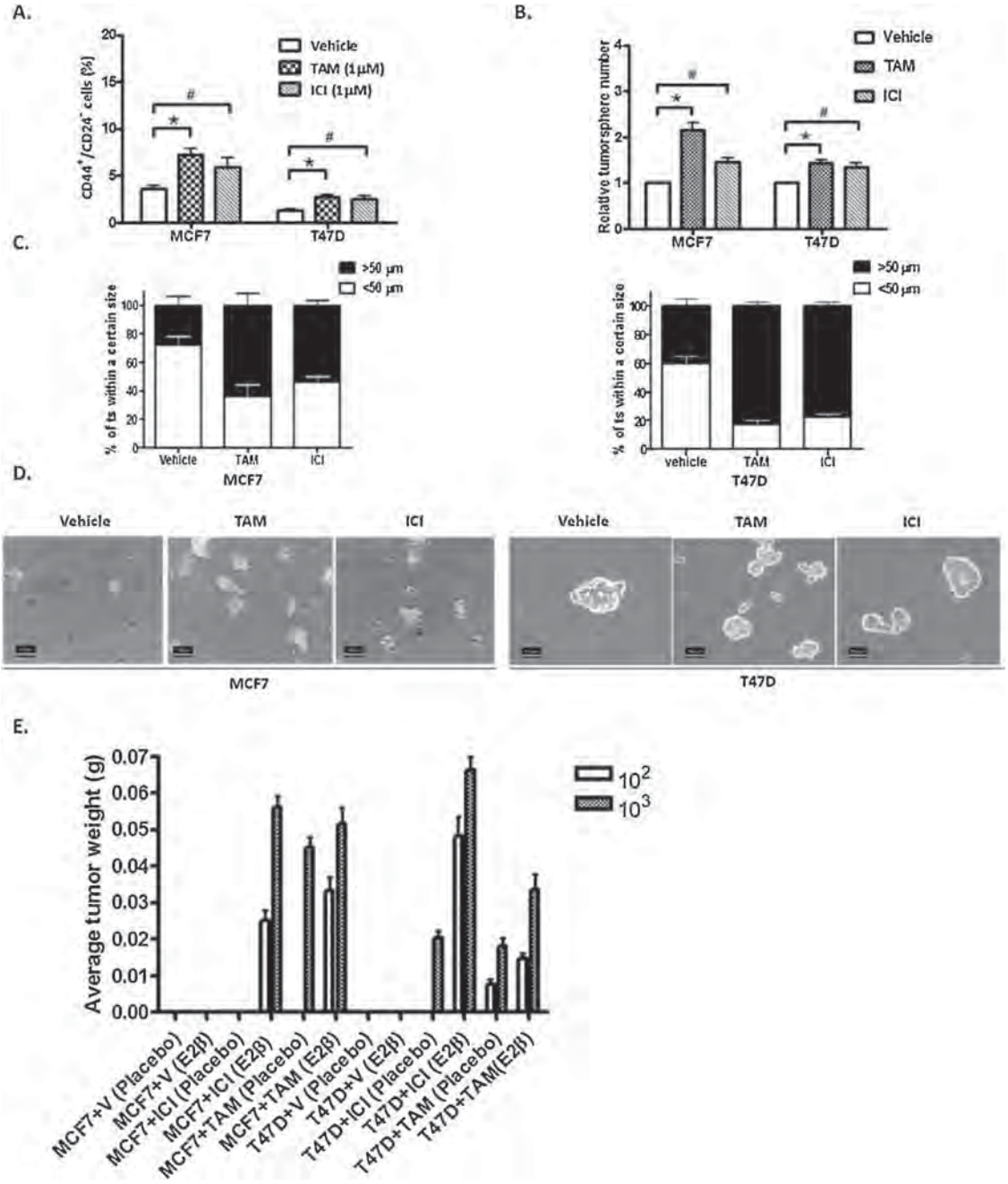

Fig. 2. Antiestrogen treatment enriched cancer stem/progenitor cells in ER-positive breast cancer cells. (A). The monolayer (parental) MCF7 and T47D cells were treated with vehicle (ethanol) or 1 μM of tamoxifen and ICI 182, 780 for seven days. The population of $CD44^+/CD24^-$ cells in the remaining cells were analyzed after staining with fluorochrome-conjugated antibodies. The columns represent the means of three experiments; bars, SE. * & #, $P < 0.05$ for vehicle treated cells vs cells treated with tamoxifen or ICI 182, 780, respectively. (B) Antiestrogen treatment increases the number of tumorspheres formed by MCF7 and T47D cells. The columns represent the means of three experiments; bars, SE. * & #, $P < 0.05$ for cells treated with vehicle vs cells treated with tamoxifen or ICI 182, 780, respectively. (C). Percentage of tumorspheres (ts) with a certain size, >50 μm or <50 μm. Antiestrogen treatment increases the number of cells from dissociated tumorspheres formed by MCF7 and T47D cells. The columns represent the means of three experiments; bars, SE. (D). Tumorsphere formation assay was used to assess the population of the cancer stem/progenitor cells in the antiestrogen treated cells. The representative results are shown. (E). Antiestrogen treatment enhances the tumor-seeding efficiency of ER-positive breast cancer cells. 1×10^2 and 1×10^3 of MCF7 and T47D cells treated with antiestrogen were implanted in the mammary fatpad of the ovariectomized female mice supplemented with estrogen or placebo pellets. The tumor-seeding efficiency was examined by measurement of tumor weight. The data represent the mean ± SE observed in six mice in each group.

cells (Fig. 1A and B). Our results thus suggested that ER-α36 is highly expressed in ER-positive breast cancer stem/progenitor cells.

To determine whether ER-positive breast cancer stem/progenitor cells are more resistant to antiestrogens, we cultured MCF7 and T47D cells under suspension conditions to form tumorspheres for five days and then different concentrations of antiestrogens were added for another five days. We found that cells from the tumorspheres are more resistant to tamoxifen and ICI 182, 780 than the parental cells; antiestrogens even enhanced cell growth at lower concentrations in tumorsphere cells (Fig. 1C and D). Our results thus suggested that ER-positive breast cancer stem/progenitor cells are more resistant to antiestrogens than the bulk breast cancer cells.

3.2. Antiestrogen treatment enriched breast cancer stem/progenitor cells from ER-positive breast cancer cells

Based on the findings described above, we reasoned that antiestrogens might kill the bulk breast cancer cells while leave the stem/progenitor cells intact. To test this possibility, we treated ER-positive breast cancer MCF7 and T47D cells with 1 μM of tamoxifen or ICI 182, 780 for seven days and the survived cells were then tested for the properties of cancer stem/progenitor cell. We found that the $CD44^+/CD24^-$ positive cell populations were significantly increased in the cells survived from antiestrogen treatment (Fig. 2A). We also tested the ability of these survived cells to form tumorspheres and found that the cells survived antiestrogen treatment formed more and bigger size tumorspheres compared to vehicle treated cells (Fig. 2B-D).

We then examined the tumor-seeding efficiency, a characteristic of the cancer stem/progenitor cells, of the cells survived antiestrogen treatment and found that ER-positive breast cancer cells survived antiestrogen treatment exhibited potent tumor-seeding efficiency; generated measureable tumors when 100 cells were used, which was not found in cells treated with vehicle (Fig. 2E). We also found that estrogen supplement enhanced tumor growth (Fig. 2E). Our results thus strongly suggested that antiestrogen treatment enriched breast cancer stem/progenitor cells that are still responsive to estrogen stimulation.

3.3. Tamoxifen resistant MCF7 cells contain high percentage of stem/progenitor cells.

Based on the finding that antiestrogen treatment enriched breast cancer stem/progenitor cells, we reasoned that antiestrogen resistant breast cancer cells might contain more stem/progenitor cells than antiestrogen sensitive cells. Previously, we established a tamoxifen resistant cell line MCF7/TAM by continuous exposure of tamoxifen sensitive MCF7 cells to 1 μM of tamoxifen for six months [17]. To assess the population of the stem/progenitor cells in the MCF7/TAM cell line, we examined the $CD44^+/CD24^-$ cell populations in parental MCF7 (MCF7/P) and MCF7/TAM cells. We found that the $CD44^+/CD24^-$ cell population was significantly increased in MCF7/TAM cells compared to the MCF7/P cells (Fig. 3A and B). Tamoxifen at 1 μM further expanded the pool of $CD44^+/CD24^-$ cells in MCF7/TAM cells (Fig. 3A and B).

We then tested the capability of these cells to form tumorspheres and found that the MCF7/TAM cells formed more and bigger tumorspheres compared to the parental MCF7 cells (Fig. 3C and D). Treatment with 1 μM Tamoxifen significantly increased the number of the tumorspheres formed by MCF/TAM cells (Fig. 3C and D). oweverThese results indicated that the tamoxifen resistant MCF7 cells have high percentage of stem/progenitor cells and tamoxifen treatment further expands the population of the cancer stem/progenitor cells.

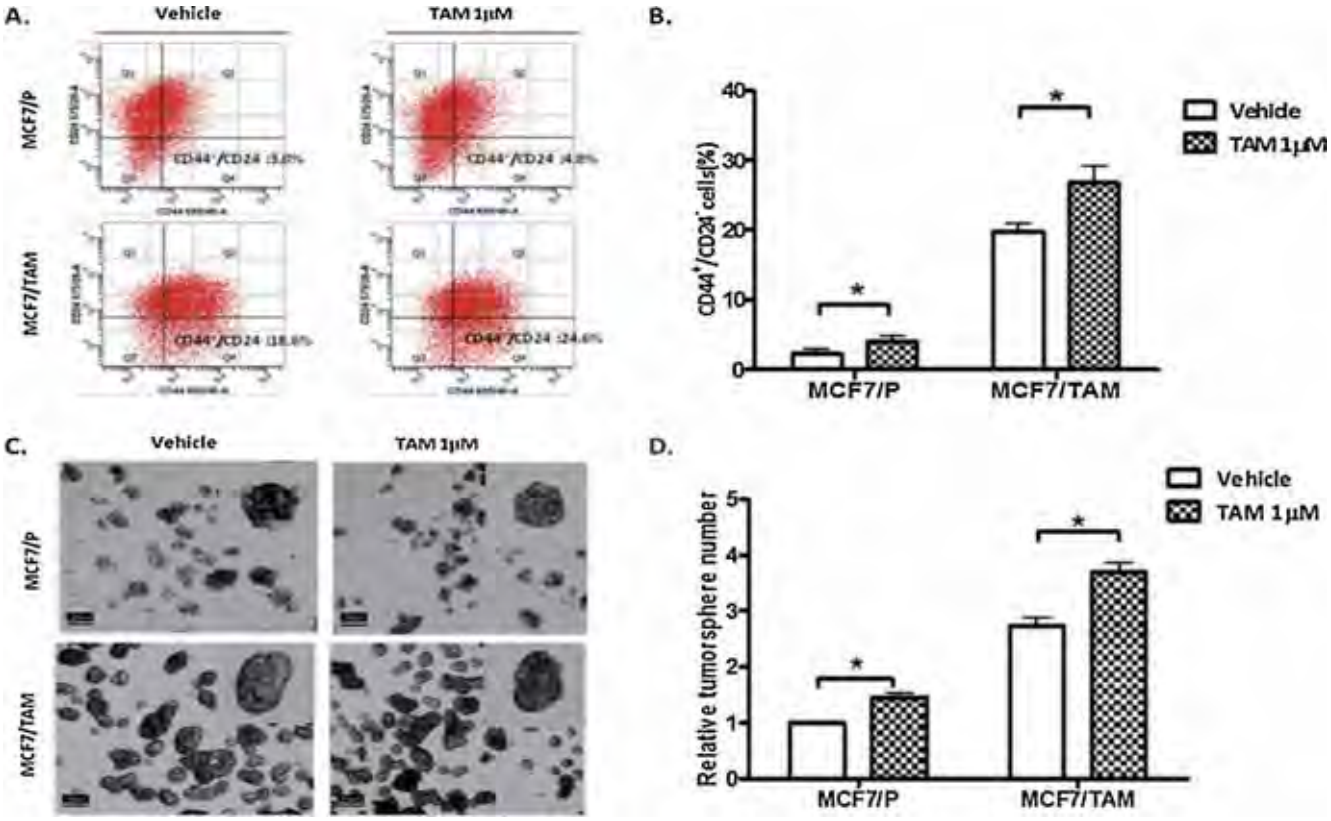

Fig. 3. Tamoxifen resistant MCF7 cells contain high percentage of cancer stem/progenitor cells. (A and B). The parental MCF7 cells (MCF7/P) and tamoxifen resistant MCF7 cells (MCF7/TAM) treated with vehicle (ethanol) or 1 μM of tamoxifen for seven days. The population of $CD44^+/CD24^-$ cells in these cells were analyzed after staining with fluorochrome-conjugated antibodies. (A). Representative experimental results are shown. (B). The columns represent the means of three experiments; bars, SE. *, $P < 0.05$ for vehicle treated cells vs cells treated with tamoxifen. (C). Tamoxifen positively regulates the size and number of tumorspheres from MCF7/TAM cells. Representative tumorspheres from MCF7/P and MCF7/TAM cells treated with vehicle or 1 μM tamoxifen for seven days. (D). The numbers of tumorspheres of MCF7/P and MCF7/TAM treated with vehicle or 1 μM tamoxifen were determined. The numbers of tumorspheres from MCF7/P cells treated with vehicle were arbitrarily set as 1. The columns represent the means of three experiments; bars, SE. *, $P < 0.05$ for cells treated with vehicle vs cells treated with tamoxifen.

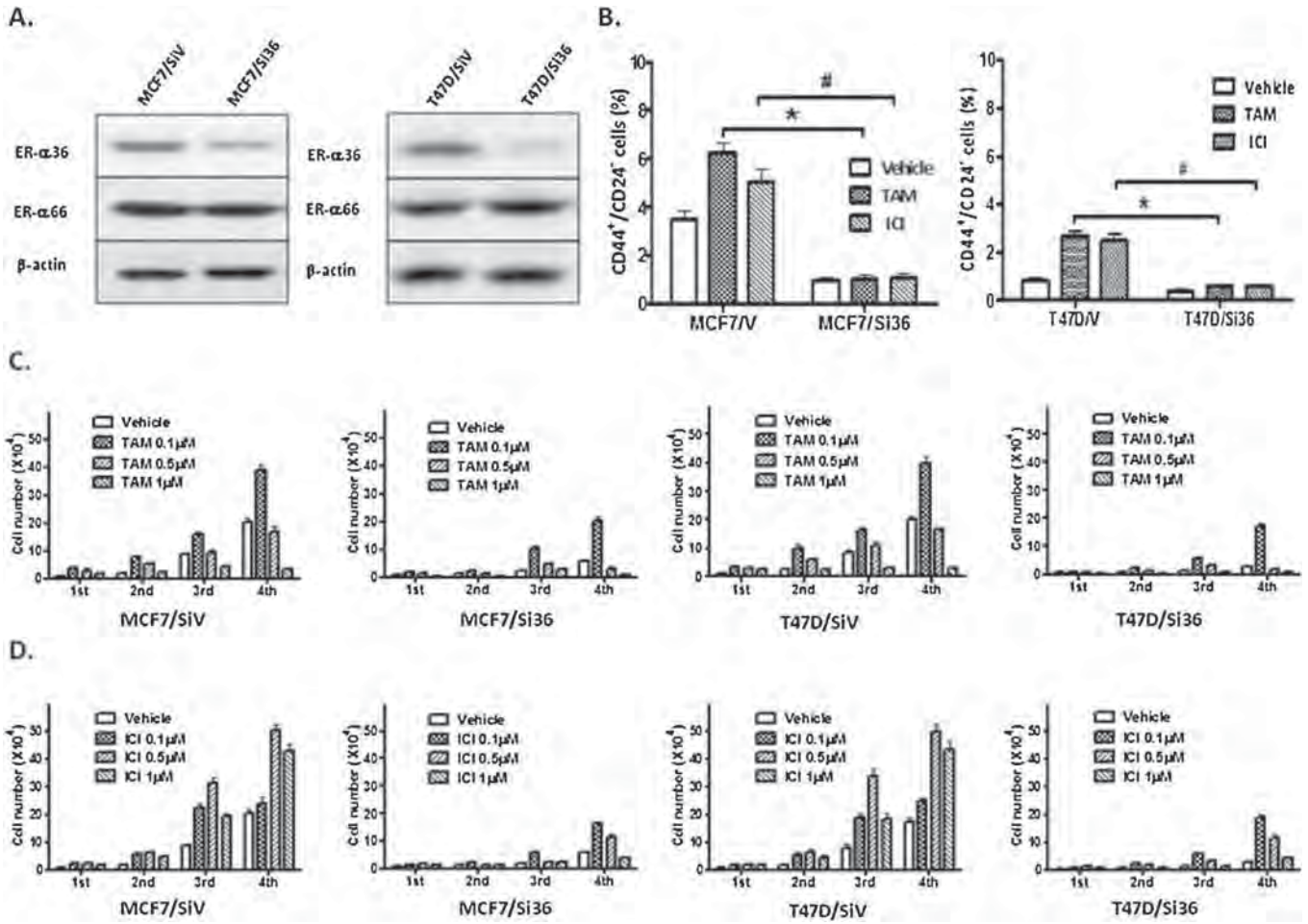

Fig. 4. ER-α36-mediated antiestrogen signaling stimulates the self-renewal of ER-positive breast cancer stem cells. (A). Western blot analysis of ER-α36 and ER-α66 expression in MCF7 and T47D control cells transfected with the empty expression vector, MCF7/SiV and T47D/SiV cells and in MCF7 and T47D cells with knocked-down levels of ER-α36 expression, MCF7/Si36 and T47D/Si36. The band density of each band relative to β-actin is shown in supplemental Fig. S1. (B). The monolayer MCF7 and T47D cell variants were treated with vehicle (ethanol) or 1 μM of tamoxifen (TAM) and ICI 182, 780 (ICI) for seven days. The populations of $CD44^+/CD24^-$ cells were analyzed after staining with fluorochrome-conjugated antibodies. The columns represent the means of three experiments; bars, SE. * and #, $P < 0.05$ for vehicle treated cells vs cells treated with tamoxifen or ICI 182, 780, respectively. (C and D). Cells variants in the presence of vehicle (ethanol) or different concentrations of tamoxifen (TAM) or ICI 182, 780 (ICI) for seven days. The cells from tumorspheres were passed once a week for four generations. The numbers of cells from dissociated tumorspheres were determined. Three dishes were used for each group and the experiments were repeated three times. The columns represent the means of three experiments; bars, SE.

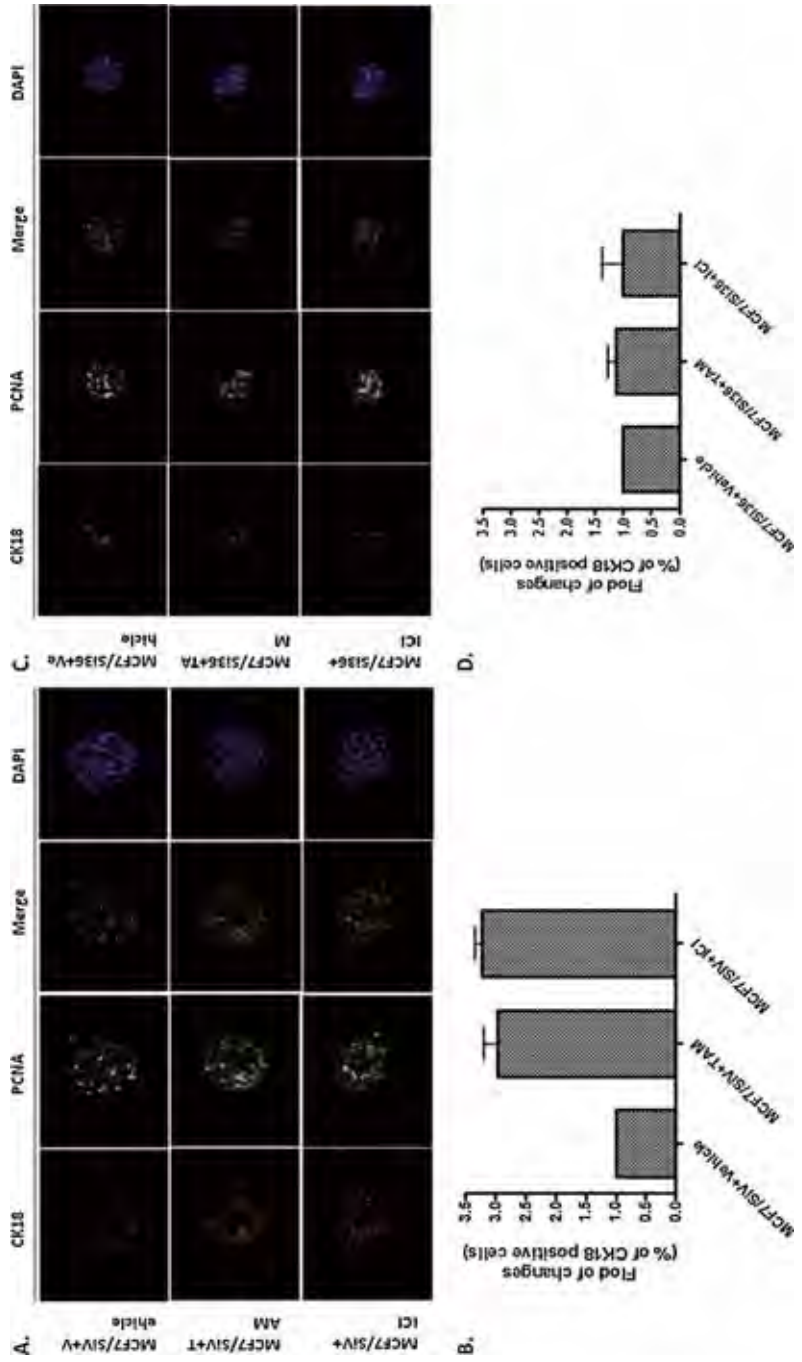

Fig. 5. ER-α36-mediated antiestrogen signaling induced proliferation of luminal epithelial lineage specific ER-positive breast cancer progenitor cells. (A and C). Indirect Immunofluorescent staining for CK18 (red) and PCNA (green) as well as merged images in MCF7/SiV and MCF7/Si36 cells treated with vehicle, tamoxifen (TAM) and ICI 182, 780 (ICI). DAPI (blue) was used to stain the nuclear region. (B and D). Percentage of CK18 positive cells in tumorspheres derived from MCF7/SiV and MCF7/Si36 cells treated with vehicle, tamoxifen (TAM) and ICI 182, 780 (ICI) treated with vehicle. The columns represent fold of changes with the percentage of CK18 positive cells treated with vehicle was arbitrarily set as 1. The experiment was repeated three times; bars, SE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. ER-α36 mediates mitogenic antiestrogen signaling in ER-positive breast cancer stem/progenitor cells

Based on our findings that ER-positive breast cancer stem/progenitor cells express high levels of ER-α36, and ER-positive breast cancer cells with high levels of ER-α36 expression are resistant to tamoxifen [17], we reasoned that ER-α36 might play a role in resistance of ER-positive breast cancer stem/progenitor cells to antiestrogens. To examine this possibility, we used MCF7 and T47D control cells transfected with an empty expression vector (MCF7/SiV and T47D/SiV) as well as their derivatives with knocked-down levels of ER-α36 expression; MCF7/Si36 and T47D/Si36 (Fig. 4A). The $CD44^+/CD24^-$ cell population in tumorspheres formed by different cell lines in the presence and absence of tamoxifen or ICI 182, 780 at 1 μM was assessed with flowcytometry. We found that in the absence of antiestrogens, MCF7/Si36 and T47D/Si36 contained a lower percentage of $CD44^+/CD24^-$ cells compared to the vector control cells (Fig. 4B), consistent with our recent report that ER-α36 plays a role in maintenance of ER-positive breast cancer stem/progenitor cells [19]. We further found both tamoxifen and ICI 182, 780 increased the populations of $CD44^+/CD24^-$ cells in MCF/SiV and T47D/SiV cells while failed to significantly increase $CD44^+/CD24^-$ cells in MCF7/Si36 and T47D/Si36 cells (Fig. 4B), suggesting ER-α36 mediates mitogenic antiestrogen signaling in ER-positive breast cancer stem/progenitor cells.

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2014.08.017.

According to the stem cell model, stem cells divide asymmetrically to maintain homeostasis of the stem cell pool, a process called self-renewal, while the growth of the bulk population relies on progenitor cells. Previously, it has been reported that breast cancer stem/progenitor cells are able to generate tumorspheres, and the tumorspheres can be re-populated after re-culture because of their self-renewal capabilities [20]. To examine whether ER-α36-mediated antiestrogen signaling also influences the self-renewal of ER-positive breast cancer stem cells, we studied the tumorsphere formation of MCF7 and T47D control cells as well as their derivatives with knocked-down levels of ER-α36 expression through serial passage in the absence or presence of antiestrogens. The cells were treated with vehicle, different concentrations of tamoxifen or ICI 182, 780 at the time of each seeding. All viable cells were determined at the end of each passage and seeded for next passage for a total of four passages. We then dissociated tumorspheres and determined the cell number. We found that the MCF7 and T47D control cells produced more tumorsphere cells in the 2nd, 3rd and 4th generations in the absence of antiestrogens while antiestrogen treatment further increased the number of tumorsphere cells in each generation (Fig. 4C and D). We also found that MCF7/Si36 and T47D/Si36 generated less tumorsphere cells in each generation compared to the control cells in the presence and absence of antiestrogens (Fig. 4C and D). Our results thus indicated that ER-α36-mediated antiestrogen signaling positively regulates the self-renewal of ER-positive breast cancer stem cells.

3.5. ER-α36-mediated antiestrogen signaling induced proliferation of luminal epithelial lineage specific ER-positive breast cancer progenitor cells

Breast cancer stem cells are able to differentiate into both luminal epithelial and myoepithelial cells [5]. Since tamoxifen at lower concentrations even expanded the populations of cancer stem/progenitor cells, we decided to investigate and compared the differentiation lineages of the stem cells derived from MCF7 derivatives in the presence of antiestrogens. Intact tumorspheres in suspension culture were treated with vehicle or 1 μM of tamoxifen or ICI 182, 780 for five days and the indirect immunofluoresces assay was performed to determine the effects of estrogen on differentiation lineages of these cells using cytokeratin 18 (CK18) for luminal epithelial cells and CD10 for myoepithelial cells. In tumorspheres formed by MCF7/SiV cells, we found that both tamoxifen and ICI 182, 780 increased the population of cells that were positive for CK18 (Fig. 5A and B). Both antiestrogens failed to increase percentage of cells expressing CK18 in MCF7/Si36 cells (Fig. 5C and D). We did not observe significant CD10 expression in these cells in the presence or absence of antiestrogens (data not shown), consistent with our recent report that ER-positive breast cancer stem cells mainly differentiated via luminal epithelial lineage [19].

To examine whether antiestrogen treatment induces differentiation of breast cancer stem cells or proliferation of breast cancer progenitor cells that express the CK18 marker, we decided to examine if the cells positive for CK18 were still proliferative. Indirect immunofluorescence staining was performed to examine the co-expression of CK18 with proliferating cell nuclear antigen

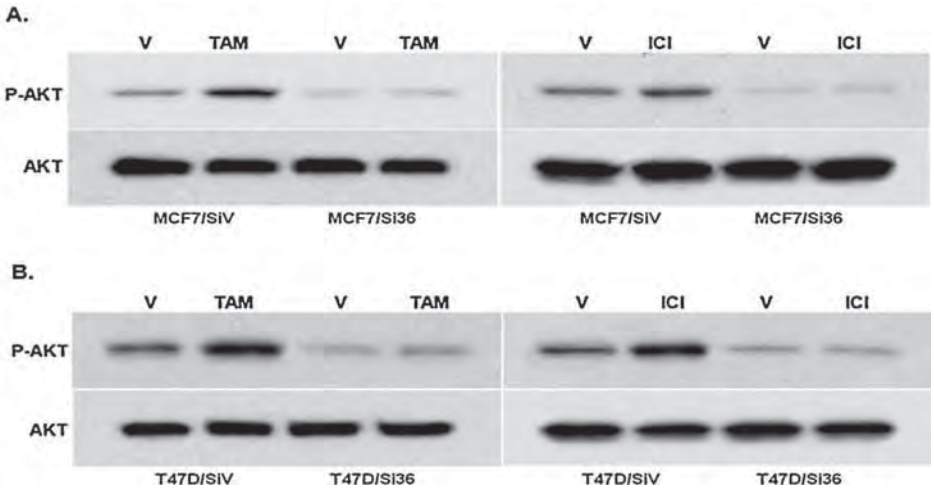

Fig. 6. The AKT signaling pathway is involved in ER-α36-mediated antiestrogen signaling of ER-positive breast cancer stem/progenitor cells. (A and B). Western blot analysis of the cell lysates from tumorspheres derived from MCF7/SiV and T47D/SiV, and MCF7/Si36 and T47D/Si36 cells treated with ethanol (vehicle); 1 μM of tamoxifen (TAM) or ICI 182, 780 (ICI) using indicated antibodies. The p-AKT band intensity relative to total AKT is shown in supplemental Fig. S2.

(PCNA), a marker for cell proliferation. We found that in MCF7/SiV cells, the percentages of both CK18 and PCNA positive cells were significantly increased in the presence of antiestrogens while in MCF7/Si36 cells, the number of cells co-expressed both PCNA and CK18 failed to increase in response to antiestrogens (Fig. 5). Our results thus strongly indicated that ER-α36 mediated antiestrogen signaling stimulates proliferation of ER-positive breast cancer progenitor or intermediate cells.

3.6. The AKT signaling pathway is involved in antiestrogen resistance of ER-positive breast cancer stem/progenitor cells

Recently, we reported that ER-α36 is involved in tamoxifen resistance by mediating tamoxifen-induced activation of the PI3K/AKT signaling pathway [17]. We decided to examine whether the AKT activation is also involved in antiestrogen resistance of ER-positive breast cancer stem/progenitor cells. We treated tumorsphere cells derived from MCF7/SiV and T47D/SiV cells with 1 μM of tamoxifen and ICI 182, 780, respectively and performed Western blot analysis using the phosphorylation specific and phosphorylation non-specific anti-AKT antibodies. We found that both antiestrogens induced the phosphorylation of the AKT in the tumorsphere cells derived from MCF7/SiV and T47D/SiV cells (Fig. 6A and B). However, the tumorsphere cells derived from MCF7/Si36 and T47D/Si36 exhibited low basal levels of AKT phosphorylation. Antiestrogens failed to induce the AKT phosphorylation in these cells (Fig. 6A and B). Our results thus suggested the ER-α36-mediated antiestrogen activation of the AKT signaling pathway is involved in antiestrogen resistance of ER-positive breast cancer stem/progenitor cells.

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2014.08.017.

4. Discussion

In this study, the breast cancer stem/progenitor cells derived from ER-positive breast cancer MCF7 and T47D cells were used as models to investigate their responses to antiestrogens. Here, we demonstrated that ER-positive breast cancer stem/progenitor cells are relatively more resistant to antiestrogens than the bulk breast cancer cells. We showed that antiestrogen treatment enriched breast cancer stem/progenitor cells and also stimulated the self-renewal of breast cancer stem cells at low concentrations. We also showed that tamoxifen resistant MCF7 cells contain high percentage of cancer stem/progenitor cells. We further demonstrated that ER-α36 plays an important role in antiestrogen resistance of ER-positive breast cancer stem/progenitor cells.

It is increasingly recognized that breast cancer has a population of cancer stem/progenitor cells that maintains tumor growth [4–6]. It is also known that cancer stem/progenitor cells are often resistant to most of the current cancer therapeutic approaches such as chemo- and radio-therapy [7–9]. Previously, it was proposed that breast cancer stem/progenitor cells may be involved in endocrine resistance [10], which was supported by a recent study that the breast cancer stem/progenitor cells induced by Sox2 introduction are resistant to tamoxifen [11]. Here, we demonstrated that ER-positive breast cancer stem/progenitor cells are relatively more resistant to antiestrogens tamoxifen and ICI 182, 780 than the bulk cells. We showed that antiestrogen treatment enriched cancer stem/progenitor cells from these ER-positive breast cancer cells and antiestrogens at low concentrations were able to induce proliferation of ER-positive breast cancer progenitor cells that expressed CK18. Since there are no specific markers to differentiate breast cancer stem, progenitor or intermediate cells (non-stem proliferative cells), it is difficult to determine which cell populations that antiestrogens really stimulated. However, the results that antiestrogen treatment increased the size of tumorspheres formed by ER-positive breast cancer cells and CK18 positive cells still underwent cell proliferation suggested that antiestrogens stimulate proliferation of breast cancer progenitor/intermediate cells.

The number of tumorspheres formed after serial passage is believed to reflect stem cell self-renewal, whereas the size of the tumorspheres formed usually reflects the rate of proliferation of more mature progenitor cells [4]. Our results showed that antiestrogens increased both the number and size of the tumorspheres formed by antiestrogen sensitive breast cancer cells as evidenced by increased number of cells dissociated from tumorspheres suggested that antiestrogens may expand the pool of breast cancer stem cells and also induced proliferation of more mature progenitor cells. Stem cells maintain self-renewal and differentiation in two ways: asymmetric and symmetric cell division [22,23]. Asymmetric cell division generates two daughter cells with different fate: one retains as a stem cell and another a differentiated cell, which maintains the pool of stem cells. In the symmetric division, however, two daughter cells produced have the same fate: both cells are either stem cells to expand the pool of stem cells or differentiated cells. Here, we found that both tamoxifen and ICI 182, 780 increased the numbers of tumorspheres from ER-positive breast cancer cells, suggesting that antiestrogens may expand the pool of cancer stem cells by increasing the symmetric division.

Furthermore, we found that ER-positive breast cancer stem/progenitor cells expressed high levels of ER-α36 and the cells with knocked-down levels of ER-α36 expression weakly increased the populations of stem/progenitor cells in response to antiestrogens. These findings are consistent with our recent report that ER-positive breast cancer cells with high levels of endogenous ER-α36 expression are relatively more resistant to tamoxifen than cells with lower ER-α36 expression [17] and ER-α36 plays an important role in positive regulation and maintenance of ER-positive breast cancer stem/progenitor cells [19]. Thus, our results suggested that high level of ER-α36 expression in ER-positive breast cancer stem/progenitor cells is one of the mechanisms by which the ER-positive breast cancer stem/progenitor cells are relatively more resistant to antiestrogens than the bulk cells.

Recently, we reported that ER-α66 protein was re-distributed from the cell nucleus to the cytoplasm and was destabilized through the proteasome degradation system in the cancer stem/progenitor cells enriched from ER-positive breast cancer MCF7 and T47D cells, which resulted in an attenuated genomic estrogen-signaling mediated by ER-α66 in the ER-positive breast cancer stem/progenitor cells [19]. Here we observed that ER-α66 is expressed a reduced level in ER-positive breast cancer stem/progenitor cells, consistent with the previous report [24]. Thus, our results suggested that the redistribution and destabilization of the ER-α66 protein in ER-positive breast cancer stem/progenitor cells make these cells behave like ER-negative cells, which provides another mechanism by which ER-positive breast cancer stem/progenitor cells become refractory to antiestrogens.

Previously, we reported that ER-α36 mediated agonist activity of tamoxifen and ICI 182, 780 [16], and both tamoxifen and ICI 182, 780 exhibited a biphasic growth response curve; stimulated cell proliferation at low concentrations and inhibited cell growth at high concentrations [16]. Recently, we also reported that ER-positive breast cancer cells with high levels of ER-α36 expression require relatively higher concentrations of tamoxifen to suppress the PI3K/AKT signaling and to inhibit cell growth [17]. Thus, ER-α36-mediated antiestrogen resistance is a concentration dependent event; high concentrations of

antiestrogens still exhibited cytotoxic activity in cells express high levels of ER-α36 such as in ER-positive breast cancer stem/progenitor cells. Thus, antiestrogen resistance is a concentration dependent event.

A better understanding of how ER-positive breast cancer stem/progenitor cells that drive breast tumor resistance to endocrine therapy is an essential step to advance our breast cancer knowledge and improve management. Our current results provided strong evidence to support an important role of ER-α36-mediated antiestrogen signaling in antiestrogen resistance of breast cancer stem/progenitor cells and provided a rational for development of therapeutic approaches to overcome antiestrogen resistance of breast cancer stem/progenitor cells by targeting ER-α36.

Acknowledgements

We thank Dr. Perry Greg for his technical support for flow cytometry. This work was supported by Department of Defense grant DAMD 11-1-0497 and by Nebraska Tobacco Settlement Biomedical Research Program Award LB595 to Z.Y. Wang. This work was also supported by National Natural Sciences Foundation of China grant No: 31240031 to M.L. Wang and No: 81272754 to L.J. Liu.

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Elsevier Editorial System(tm) for The Journal of Steroid Biochemistry and Molecular Biology

Manuscript Draft

Manuscript Number: SBMB-D-14-00306

Title: Downregulation of ER-alpha36 Expression Sensitizes HER2 Overexpressing Breast Cancer Cells to Tamoxifen

Article Type: Full Length Article

Keywords: ER-alpha36; EGFR; HER2; breast cancer; tamoxifen resistance

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Abstract: Tamoxifen provided a successful treatment for ER-positive breast cancer for many years. However, HER2 overexpressing breast cancer cells respond poorly to tamoxifen therapy presumably by pass. The molecular mechanisms underlying development of tamoxifen resistance have not been well established. Recently, we reported that breast cancer cells with high levels of ER-alpha36, a variant of ER-alpha, were resistant to tamoxifen and knockdown of ER-alpha36 expression in tamoxifen resistant cells with the shRNA method restored tamoxifen sensitivity, indicating that gained ER-alpha36 expression is one of the underlying mechanisms of tamoxifen resistance. Here, we found that tamoxifen induced expression of ER-alpha36-EGFR/HER2 positive regulatory loops and tamoxifen resistant MCF7 cells (MCF7/TAM) expressed enhanced levels of the loops. Disruption of the ER-alpha36-EGFR/HER2 positive regulatory loops with the dual tyrosine kinase inhibitor Lapatinib or ER-alpha36 down-regulator Broussoflavonol B in tamoxifen resistant MCF7 cells restored tamoxifen sensitivity. In addition, we also found both Lapatinib and Broussoflavonol B increased the growth inhibitory activity of tamoxifen in tumorsphere cells derived from MCF7/TAM cells. Our results thus demonstrated that elevated expression of the ER-alpha36-EGFR/HER2 loops is one of the mechanisms by which ER-positive breast cancer cells escape tamoxifen therapy. Our results thus provided a rational to develop novel therapeutic approaches for tamoxifen resistant patients by targeting the ER-alpha36-EGFR/HER2 loops.

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Cover Letter

September 10, 2014.
Editor,
The Journal of Steroid Biochemistry and Molecular Biology

Dear Editor:

I am submitting our manuscript entitled “Downregulation of ER-α36 Expression Sensitizes HER2 Overexpressing Breast Cancer Cells to Tamoxifen” by Li Yin, Xiaohua Pan, Xin-Tian Zhang, Yu-Ming Guo, Zhao-Yi Wang, Yaoqin Gong and Molin Wang. We believe the results described in this manuscript are significant and will appeal to the wide readership of The Journal of Steroid Biochemistry and Molecular Biology. These data have not been published previously or in any other journals.

Several years ago, Dr. Wang et al. identified and cloned a 36kDa variant of ER-α, ER-α36, which is mainly expressed on the plasma membrane and mediates non-genomic estrogen signaling (Wang et al., PNAS 2006, 103: 9063–9068). Our present study is investigating the effects of the ER-α36-HER2 positive regulatory loop in tamoxifen resistance. Tamoxifen provided a successful treatment for ER-positive breast cancer for many years. However, HER2 overexpressing breast cancer cells respond poorly to tamoxifen therapy. The molecular mechanisms underlying development of tamoxifen resistance have not been well established. In this study, we found that tamoxifen induced expression of ER-α36-EGFR/HER2 positive regulatory loops and tamoxifen resistant MCF7 cells (MCF7/TAM) expressed enhanced levels of the loops. Disruption of the ER-α36-EGFR/HER2 positive regulatory loops with the dual tyrosine kinase inhibitor Lapatinib or ER-α36 down-regulator Broussoflavonol B in tamoxifen resistant MCF7 cells restored tamoxifen sensitivity. In addition, we also found both Lapatinib and Broussoflavonol B increased the growth inhibitory activity of tamoxifen in tumorsphere cells derived from MCF7/TAM cells. Our results thus demonstrated that elevated expression of the ER-α36-EGFR/HER2 loops is one of the mechanisms by which ER-positive breast cancer cells escape tamoxifen therapy. These new concepts will have major impact in areas of basic and clinical breast cancer research.

Given the groundbreaking nature and broad implications of the concepts that are contained in our manuscript, we feel strongly that this paper deserves to be published in *SBMB*.

Sincerely
Yours Sincerely，
Molin Wang, Ph.D.
Associate Professor of Medical Genetics,
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Downregulation of ER-α36 Expression Sensitizes HER2 Overexpressing Breast Cancer Cells to Tamoxifen

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Short Title: ER-α36 in tamoxifen resistance of HER2 overexpressing breast cancer cells.
Key Words: ER-α36, EGFR, HER2, breast cancer, tamoxifen resistance.

This work was supported by Department of Defense grant DAMD 11-1-0497 and Nebraska Tobacco Settlement Biomedical Research Program Awards (LB-595) to Z.Y. Wang. This work was also supported by National Natural Science Foundation of China (31240031) and Natural Science Foundation of Shandong Province of China (ZR2012HQ016) to M Wang.

Abstract

Tamoxifen provided a successful treatment for ER-positive breast cancer for many years. However, HER2 overexpressing breast cancer cells respond poorly to tamoxifen therapy presumably by pass. The molecular mechanisms underlying development of tamoxifen resistance have not been well established. Recently, we reported that breast cancer cells with high levels of ER-α36, a variant of ER-α, were resistant to tamoxifen and knockdown of ER-α36 expression in tamoxifen resistant cells with the shRNA method restored tamoxifen sensitivity, indicating that gained ER-α36 expression is one of the underlying mechanisms of tamoxifen resistance. Here, we found that tamoxifen induced expression of ER-α36-EGFR/HER2 positive regulatory loops and tamoxifen resistant MCF7 cells (MCF7/TAM) expressed enhanced levels of the loops. Disruption of the ER-α36-EGFR/HER2 positive regulatory loops with the dual tyrosine kinase inhibitor Lapatinib or ER-α36 down-regulator Broussoflavonol B in tamoxifen resistant MCF7 cells restored tamoxifen sensitivity. In addition, we also found both Lapatinib and Broussoflavonol B increased the growth inhibitory activity of tamoxifen in tumorsphere cells derived from MCF7/TAM cells. Our results thus demonstrated that elevated expression of the ER-α36-EGFR/HER2 loops is one of the mechanisms by which ER-positive breast cancer cells escape tamoxifen therapy. Our results thus provided a rational to develop novel therapeutic approaches for tamoxifen resistant patients by targeting the ER-α36-EGFR/HER2 loops.

Introduction:

Human epidermal growth factor receptor 2 (ERBB2/HER2) is amplified and/or overexpressed in approximately 15-20% of breast cancers (Chia et al., 2008; Slamon et al., 1987). HER2-overexpressing breast cancer patients exhibit a poor prognosis because of a high incidence of metastases, disease progression, and resistance to current endocrine therapy regimens in the tumors co-expressing estrogen receptor (ER) (Pegram et al., 1998; Slamon et al., 1987; Wright et al., 1989). Clinically, the current therapy for HER2 expressing, early-stage and metastatic breast cancer patients employs a combination of HER2-targeted monoclonal antibody (Trastuzumab, Herceptin) treatment with chemotherapy (Docetaxel or Vinorelbine) (Hoeferlin et al., 2013). Additionally, the dual inhibitor of EGFR (ERBB1)/HER2 (ERBB2) receptor, Laptinib, is also used as a combination treatment with Capecitabine for HER2-positive advanced breast cancer that has progressed after previous treatment with other chemotherapies or combinational therapies (Hoeferlin et al., 2013). However, 60% of metastatic breast cancers that express HER2 fail to respond to current available anti-HER2 therapies (Burstein et al., 2001; Marty et al., 2005; Murphy and Morris, 2012). Trastuzumab/Lapatinib combination treatment has provided significant benefits to patients; 50% of patients to the combination therapy whereas 32-43% with Trastuzumab alone, but there is still a large percentage (50%) of patients that do not respond (Slamon et al., 2001). Thus, novel therapeutic approaches are urgently needed for the patients with HER2 positive breast cancer but fail to respond to anti-HER2 therapies.

Estrogen signaling through its cognate receptor (ER) plays an important role in breast cancer tumorigenesis and biology. Antiestrogen therapy has been widely used for treatment of ER-positive breast cancer. However, in clinical practice, it has been observed that HER2 overexpressing tumors, even co-expressing ER, have reduced responsiveness to antiestrogen therapy (DiGiovanna, 1999a, b). Hence, enhanced HER2 expression stimulates growth factor signaling that can rescue estrogen-

dependent breast cancer cells from the effects of estrogen deprivation.

The selective estrogen-receptor modulator tamoxifen is the most widely used antiestrogen in clinical practice. Previously, it has been reported that overexpression of HER2 in estrogen-dependent and tamoxifen sensitive cells resulted in tamoxifen resistance while a HER tyrosine kinase inhibitor restored tamoxifen sensitivity to these cells (Kurokawa and Arteaga, 2001). In preclinical studies, a combination of trastuzumab and tamoxifen treatment has been demonstrated to result in synergistic growth inhibition of HER2 expressing breast cancer cells (Argiris et al., 2004; Kunisue et al., 2000; Wang et al., 2005a; Witters et al., 1997). The dual kinase inhibitor, Lapatinib, also has been shown to cooperate with tamoxifen to inhibit cell proliferation and estrogen dependent gene expression in antiestrogen-resistant breast cancer (Chu et al., 2005). Thus, combination of HER2 and ER targeted therapies may provide a novel and effective approach to treatment of HER2-overexpressing, tamoxifen resistant breast cancer. The molecular mechanism underlying the synergistic interaction between tamoxifen and trastuzumab has not been well established, which might influence further development of this therapeutic approach.

Previously, our laboratory identified and cloned a variant of ER-α, ER-α36, which has a molecular weight of 36-kDa (Wang et al., 2005b, 2006). The transcript of ER-α36 is initiated from a previously unidentified promoter in the first intron of the ER-α gene (Zou et al., 2009). This ER-α differs from the original 66 kDa ER-α (ER-α66) because it lacks both transcriptional activation domains (AF-1 and AF-2) but retains the DNA-binding and dimerization domains, and partial ligand-binding domain (Wang et al., 2006). ER-α36 is mainly localized near the plasma membrane and mediates membrane-initiated estrogen signaling (Wang et al., 2006). Previously, we reported the existence of a cross-regulatory loop between ER-α36 and HER2 (Kang et al., 2011); ER-α36 positively regulates HER2 expression while HER2 up-regulates the promoter activity of ER-α36

through an Ap1 site located in the promoter region of ER-α36. We also found that the breast cancer patients with tumors expressing high levels of ER-α36 less benefited from tamoxifen therapy than those with low levels of ER-α36 expression and ER-α36 expression is well correlated with HER2 expression in tumor samples (Zhang et al., 2011), suggesting that gained the ER-α36/HER2 positive regulatory loop is one of the underlying mechanisms of tamoxifen resistance. In addition, we also found a positive feedback loop between ER-α36 and EGFR (Shi et al., 2009). ER-α36 is able to mediate agonist activity of tamoxifen such as activation of the MAPK/ERK and the PI3K/AKT signaling pathways (Lin et al., 2010; Zhang et al., 2012) and is involved in development of tamoxifen resistance (Zhang and Wang, 2013).

Based on these observations, we hypothesized that the positive regulatory loop between ER-α36 and HER2 is involved in tamoxifen resistance of HER2 overexpressing breast cancer cells. Thus, disruption of this loop may restore tamoxifen sensitivity in tamoxifen resistant cells. Using HER2 overexpressing ER-positive breast cancer BT474 cells and MCF7/HER2-18 cells as models, we investigated the effects of disruption of the ER-α36-HER2 positive regulatory loop in tamoxifen resistance.

Methods:

Chemicals and antibodies

Tamoxifen was purchased from Sigma Chemical Co. (St. Louis, MO). Broussoflavonol B was obtained from Shenogen Pharma Group (Beijing, P.R. China). Anti-phospho-EGFR (Tyr1045) and –HER2/ErbB2 (Tyr1221/1222) as well as anti-EGFR and –HER2/ErbB2 (D8F12) antibodies were purchased from Cell Signaling Technology (Boston, MA). Antibodies of ER-α66 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-ER-α36 antibody was generated and characterized as described before (Zhang et al., 2012).

Cell culture and establishment of stable cell lines

MCF7 and BT474 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA). MCF7/HER2-18 cells were kindly provided by Dr. Jian Huang at Medical College of Wisconsin. All cells were maintained at 37^0C in a 10% CO_2 atmosphere in IMEM without phenol red plus 10% fetal calf serum.

To examine cell growth in the presence or absence of tamoxifen, cells maintained for three days in phenol red-free DMEM plus 2.5% dextran-charcoal-stripped fetal calf serum (HyClone, Logan, UT) were treated with different concentrations of tamoxifen, or ethanol vehicle as a control. The cells were seeded at 1 X 10^4 cells per dish in 60mm dishes and the cell numbers were determined using the ADAM automatic cell counter (Digital Bio., Korea) after seven days. Five dishes were used for each treatment and experiments were repeated more than three times.

Western blot analysis

For immunoblot analysis, cells washed with PBS were lysed with the lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 0.25 mM EDTA pH8.0, 0.1% SDS, 1% Triton X-100, 50 mM NaF) supplemented with protease and phosphatase inhibitors (Sigma). The protein amounts were measured using the DC protein assay kit (BIO-RAD Laboratories, Hercules, CA). The same amounts of the cell lysates were boiled for five minutes in loading buffer and separated on a SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane. The membranes were probed with various primary antibodies, HRP-conjugated secondary antibodies, and visualized with enhanced chemiluminescence (ECL) detection reagents (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Tumorsphere formation and flow cytometry analysis

To establish tumorspheres, cells were seeded onto Corning Ultra-Low Attachment 6-well plate (Corning Incorporated, CA) at 10,000 cells/ml and cultured seven days in the tumorsphere medium:

phenol-red free DMEM/F12 medium (Invitrogen) supplemented with 1 X B-27 (Invitrogen), 20 ng/ml epidermal growth factor (Sigma-Aldrich) and 20 ng/ml basic fibroblast growth factor (ProSpec, NJ), 0.5μg/mL hydrocortisone (Sigma). Tumorspheres were collected, washed with PBS, and incubated with Trypsin-EDTA (0.25%/0.5 mM) for two minutes at 37°C to dissociated cells. The number of tumorspheres and dissociated cells were counted using a Multisizer 3 Coulter Counter (Beckman Coulter, Brea, CA) and the ADAM automatic cell counter, respectively. For tamoxifen treatment assays, tumorspheres were treated with tamoxifen or vehicle (ethanol) as a control. Three dishes were used for each group and all experiments were repeated three times.

For $CD44^+/CD24^-$ cell analysis, single cell suspension washed with cold PBS/1% BSA were incubated with PerCP-Cy^{TM}5.5 mouse anti-human CD44 and PE mouse anti-human CD24 in PBS/1% BSA for 30 minutes at 4°C. After incubation, the cells were washed twice in cold PBS/1% BSA and re-suspended in cold PBS/1% BSA for flow cytometry analysis.

Statistical analysis

Data were summarized as the mean ± standard error (SE) using the GraphPad InStat software program. Tukey-Kramer Multiple Comparisons Test was also used, and the significance was accepted for $P < 0.05$.

Results

Enhanced ER-α36 and EGFR expression in ER-positive breast cancer BT474 cells.

BT474 is a human breast cancer cell line that is positive for ER and is estrogen dependent. BT474 highly expresses HER2 in association with gene amplification (Zhang and Wang, 2013). Previously, our laboratory identified and cloned a 36 kDa variant of ER-α, ER-α36 that functions differently from the 66 kDa full-length ER-α, ER-α66 (Wang et al., 2006). Compared to ER-positive breast cancer MCF7 cells, we found that the steady state level of ER-α36 protein was increased in

BT474 cells accompanied by upregulated HER2 and EGFR expression (Fig. 1A). We also examined ER-α36 expression in the MCF7/HER2-18 cell line, a cell line generated by stable transfection of a HER2 expression vector (Lasfargues et al., 1978) and found that ER-α36 and EGFR expression is also upregulated in MCF7/HER2-18 cells compared to MCF7 cells (Fig. 1B). Our results suggested that the positive regulatory loops of ER-α36 and EGFR/HER2 observed previously also exist in HER2 expressing breast cancer cells.

We then examined the sensitivity of these HER2 expressing cells to tamoxifen. Cells were treated with different concentrations of tamoxifen for seven days and then the survived cells were counted. We found both BT474 and MCF7/HER2-18 cells are relatively more resistant to tamoxifen compared to MCF7 cells (Fig. 1C), consistent with the concept that HER2 overexpression confers tamoxifen resistance in ER-positive breast cancer cells.

ER-α36 knock-down sensitizes HER2-expressing cells to tamoxifen

Previously, we reported ER-α36 is involved in tamoxifen resistance of ER-positive breast cancer cells (Shi et al., 2009; Zhang and Wang, 2013). To examine whether ER-α36 is also involved in tamoxifen insensitivity of HER2 -expressing breast cancer cells, we transiently transfected HER2-expressing cells with an ER-α36 specific shRNA expression vector and found the shRNA expression vector efficiently knocked down ER-α36 expression in these HER2-expressing cells while had no effect on ER-α66 expression compared to the empty vector transfected cells (Fig. 2A & C). We also observed that the expression levels of both HER2 and EGFR were also downregulated (Fig. 2A & C), suggesting the existence of the positive regulatory loops between ER-α36 and HER2/EGFR we previously reported (Kang and Wang, 2010; Zhang et al., 2011).

We then examined the sensitivity of the cells with knocked-down levels of ER-α36 to

tamoxifen and found that these cells were relatively more sensitive to tamoxifen compared to the vector transfected cells (Fig. 2B & D), consistent with our previous report that increased level of ER-α36 expression is one of the underlying mechanisms of TAM resistance and knock-down of ER-α36 expression restored TAM sensitivity in MCF7/TAM cells (Zhang and Wang, 2013). Our results thus suggested that ER-α36 is also involved in tamoxifen resistance of HER2-expressing ER-positive breast cancer cells and disruption of the ER-α36 and EGFR/HER2 regulatory loops may restore tamoxifen sensitivity in these HER2-expressing cells.

Dual kinase inhibitor Lapatinib downregulates ER-α36 expression and sensitizes HER2-expressing cells to tamoxifen.

We then sought to examine whether disruption of the ER-α36 and EGFR/HER2 regulatory loops restores tamoxifen sensitivity in these HER2-expressing cells. We first treated MCF7/HER2-18 cells with different concentrations of Lapatinib and the level of ER-α36 expression was examined with Western blot analysis. We found that Lapatinib inhibited phosphorylation of both EGFR and HER2 effectively (Supplement Figure S1) and also downregulated ER-α36 expression in MCF7/HER2-18 cells (Fig. 3A). Lapatinib treatment significantly increased sensitivity to tamoxifen in MCF7/HER2-18 cells (Fig. 3B). Similar results were also obtained in BT474 cells (Fig. 3 C & D). Taken together, these results demonstrated that the dual kinase inhibitor Lapatinib was able to disrupt the ER-α36-EGFR/HER2 positive regulatory loops and restored tamoxifen sensitivity in these HER2-expressing cells.

ER-α36 disruptor Broussoflavonol B also downregulates EGFR/HER2 expression and restores tamoxifen sensitivity

Previously, we found that the classic ER disruptor ICI 182, 780 failed to destabilize ER-α36

protein (Kang and Wang, 2010). We later discovered a novel ER-α36 disruptor Broussoflavonol B (5,7,3',4'-Tetrahydroxy-3-methoxy-6,8-diprenylflavone) purified from the bark of Broussonetia papyrifera that specifically and effectively downregulated the steady state levels of ER-α36 protein (Guo et al., 2013a; Guo et al., 2013b). We then examined whether the ER-α36 disruptor Broussoflavonol B (BB) is also able to disrupt the ER-α36-EGFR/HER2 loops and restores tamoxifen sensitivity in HER2-expressing cells. We treated HER2-expressing cells with different concentrations of BB and the expression levels of ER-α36, EGFR and HER2 were examined with Western blot analysis. We found that BB potently down-regulated ER-α36 expression but modestly downregulated expression levels of EGFR and HER2 proteins in MCF7/HER2-18 cells (Fig. 4A). BB treatment, however, strongly down-regulated ER-α36, EGFR and HER2 expression in BT474 cells (Fig. 4C). BB treatment significantly sensitized these HER2 expressing cells to tamoxifen (Fig. 4B & D). Our results thus demonstrated that ER-α36 downregulator BB was also able to disrupt the ER-α36-EGFR/HER2 positive regulatory loops and restored tamoxifen sensitivity in HER2-overexpressing cells.

ER-α36 knock-down reduces the populations of breast cancer stem/progenitor cells in these HER2-expressing cells

Recently, we found that ER-positive breast cancer stem/progenitor cells express higher levels of ER-α36 and ER-α36 plays an important role in maintenance of breast cancer stem/progenitor cells (Deng et al., 2014a; Deng et al., 2014b). In addition, tamoxifen resistant breast cancer cells contain high percentage of breast cancer stem/progenitor cells (Piva et al., 2014). We sought to investigate the function of ER-α36 in these HER2-expressing breast cancer stem/progenitor cells. We transiently transfected HER2-expressing cells with the ER-α36 specific shRNA expression vector and examined the $CD44^{+}/CD24^{-}$ phenotype cell populations. We found that ER-α36 knock-down significantly

reduced the percentages of the $CD44^{+}/CD24^{-}$ cells from these HER2 expressing cells (Fig. 5A & B). We also tested the ability of these HER2-expressing cells to form tumorspheres and the cells were cultured in the tumorsphere media and under suspension condition to form tumorspheres. We found that the HER2 expressing cells formed more tumorspheres compared to MCF7 cells while ER-α36 knocked-down cells significantly decreased tumorsphere numbers (Fig. 5 C & D). Our results thus indicated that ER-α36 plays a critical role in maintenance of HER2-expressing breast cancer stem/progenitor cells.

We also used Western blot analysis to assess expression of ER-α66, ER-α36, EGFR and HER2 in tumorsphere and attached cells. We found that ER-α36, EGFR and HER2 were all highly expressed in tumorsphere cells derived from MCF7 cells compared to attached bulk cells while ER-α66 expression was down-regulated (Fig. 6A), consistent with our previous report (Deng et al., 2014b). HER2 expressing cells exhibited enhanced basal levels of ER-α36, EGFR and HER2 expression in bulk cells, which was modestly increased in tumorsphere cells derived from these cells (Fig. 6A).

We then sought to examine whether Lapatinib and Broussoflavonol B are still able to disrupt the ER-α36-EGFR/HER2 regulatory loops in ER-positive breast cancer stem/progenitor cells. Lapatinib and BB treatment again downregulated EGFR and ER-α36 in both HER2-expressing cell lines while only slightly downregulated HER2 expression in MCF7/HER2-18 cells that were stably transfected with an HER2 expression vector. The results thus demonstrated that there exist ER-α36-EGFR/HER2 regulatory loops in ER-positive stem/progenitor cells derived from HER2 expressing breast cancer cells and both Lapatinib and Broussoflavonol B were able to disrupt the ER-α36-EGFR/HER2 regulatory loops in these breast cancer stem/progenitor cells.

Disruption of ER-α36-EGFR/HER2 positive regulatory loops sensitizes HER-expressing breast cancer

stem/progenitor cells to tamoxifen

Next, we sought to examine whether disruption of the ER-α36-EGFR/HER2 positive regulatory loops will sensitize HER2-expressing breast cancer stem/progenitor cells to tamoxifen. We cultured these HER2-expressing cells under suspension conditions to form tumorspheres for five days and then different concentrations of tamoxifen together with Lapatinib (LAP, 5 μM) or Broussoflavonol B (BB, 5 μM) were added for another five days. We found that in the presence of Lapatinib or Broussoflavonol B, the tumorspheres formed by HER2 expressing cells became sensitive to tamoxifen; tamoxifen reduced the number of tumorspheres (Fig. 6D-F).

Discussion

Endocrine therapy using antiestrogen tamoxifen is the most effective treatment for advanced ER-positive breast cancer for four decades. Tamoxifen acts through ER pathway, which has been proven to reduce relapse, death rates and risk of contralateral breast cancer. However, patients often develop resistance tamoxifen, which limit its effectiveness. Many researches were conducted to understand the molecular pathways involved in tamoxifen resistance and have revealed that multiple signaling molecules and pathways such as EGFR and HER2 are implicated in tamoxifen resistance (Normanno et al., 2005; Osborne and Schiff, 2011). All these pathways often bypass the requirement of estrogen signaling for growth of ER-positive breast cancer cells.

Both experimental and clinical evidence have indicated that the HER2 signaling pathway interacts with the estrogen-signaling pathway. Experimental evidence has shown that estrogen-dependent MCF7 cells that over express HER2 are rendered tamoxifen resistant and have reduced numbers of ER(Normanno et al., 2005; Osborne and Schiff, 2011). Hence the HER2 pathway has been investigated for its contribution towards development of tamoxifen resistance and HER2 has been proposed as a potential marker of tamoxifen sensitivity. Many clinical studies have found an

association between HER2 overexpression and tamoxifen failure (Berry et al., 2000; Bianco A. R., 1998; Borg et al., 1994; Carlomagno et al., 1996; Elledge et al., 1998; Leitzel et al., 1995; McCann et al., 1991; Ravdin P. M., 1998; Yamauchi et al., 1997). Thus, the combination therapy by targeting both HER2 and ER-α was hypothesized and tested in preclinical studies. The combination of tamoxifen and anti-HER2 antibody exhibits strong synergistic inhibition of growth in HER2-expressing ER-positive breast cancer cells (Argiris et al., 2004; Wang et al., 2005a). Chu et al., also reported that the dual kinase inhibitor Laptinib cooperates with tamoxifen to inhibit cell proliferation in antiestrogen resistant breast cancer (Chu et al., 2005).

Previously, we reported that breast cancer patients with tumors expressing high levels of endogenous ER-α36 less benefited from tamoxifen therapy than those with low levels of ER-α36 expression (Shi et al., 2009), suggesting elevated expression of ER-α36 is a mechanism underlying acquired tamoxifen resistance. Recently, we confirmed that elevated ER-α36 expression is involved in tamoxifen resistance through mediating agonist activity of tamoxifen (Zhang and Wang, 2013). We also reported that ER-α36 expression is highly correlated with HER2 expression and there are positive regulatory loops between ER-α36 and EGFR as well as ER-α36 and HER2; EGFR signaling induces the promoter activity of ER-α36 via an Ap1-binding site and ER-α36 stabilizes the EGFR protein (Kang et al., 2011; Shi et al., 2009; Zhang et al., 2011). HER2 signaling also activates ER-α36 promoter activity and ER-α36-mediated estrogen signaling induces HER2 promoter activity (Kang et al., 2011). Here, we showed that knock-down of ER-α36 expression downregulated both HER2 and EGFR expression in HER-overexpressing BT474 cells while only modestly downregulated HER2 expression in MCF7/HER2-18 cells that were stably transfected with a HER2 expression vector, consistent with our previous reports that ER-α36 modulates HER2 promoter activity (Kang et al.,

2011). In addition, an experiment with a proteasome inhibitor MG132 in MCF7/HER2-18 cells with knocked-down levels of ER-α36 expression showed that MG132 treatment restored the steady state levels of HER2 protein (Supplement Figure S2), suggesting ER-α36 also modulates the steady state levels of HER2 protein presumably through the proteasome system. Taken together, our results demonstrated that the ER-α36-EGFR/HER2 positive regulatory loops are involved in tamoxifen resistance of HER2 overexpressing ER-positive breast cancer cells. Enhanced expression of HER2 and EGFR render the cell bypass the requirement of estrogen for cell proliferation.

In the current study, we observed that inhibition of both EGFR and HER2 signaling pathways with the dual kinase inhibitor Lapatinib disrupted the positive regulatory loops, downregulated ER-α36 expression and restored tamoxifen sensitivity. Our results thus are in good agreement with the previous reports that Lapatinib restores antiestrogen sensitivity in breast cancer cells with acquired endocrine resistance (Chu et al., 2005; Leary et al., 2010). Here, we also observed that Lapatinib downregulated EGFR, HER2 and ER-α36 in BT474 cells, indicating the existence of the positive regulatory loops. Our data thus provided a novel molecular mechanism to the function of the dual kinase inhibitor Lapatinib; disruption of the ER-α36-EGFR/HER2 positive regulatory loops, which restores tamoxifen sensitivity.

Previously, we found that the potent ER-α disruptor ICI 182, 780 failed to degrade ER-α36 due to the lacking of the critical Helix 12 in the C-terminal of ER-α36 protein (Kang and Wang, 2010). Recently, we found that a falconoid, Broussoflavonol B (5, 7, 3', 4'-Tetrahydroxy-3-methoxy- 6,8-diprenylflavone) purified from the bark of Broussonetia papyrifera was able to down-regulate ER-α36 expression and inhibits proliferation of ER-positive and -negative breast cancer cells (Guo et al., 2013a; Guo et al., 2013b). Here we showed that Broussoflavonol B was also able to disrupt the ER-α36-

EGFR/HER2 positive regulatory loops; downregulated ER-α36, HER2 and EGFR, which restored tamoxifen sensitivity in HER2 expressing cells. Thus, further development of chemical compounds like Broussoflavonol B may provide novel approaches to restore tamoxifen sensitivity in HER2 expressing cells.

Accumulating experimental and clinical evidence indicate that breast cancer arises from mammary stem/progenitor cell populations (Charafe-Jauffret et al., 2009; Dontu et al., 2003; Oliveira et al., 2010). Although the possible involvement of breast cancer stem/progenitor cells in tamoxifen resistance has been proposed (O'Brien et al., 2009) and demonstrated (Piva et al., 2014), the exact function and the underlying mechanism of breast cancer stem/progenitor cells in TAM resistance remain largely unknown. Recently, we found that ER-positive breast cancer stem/progenitor cells express higher levels of ER-α36 and were more resistant to tamoxifen than the bulk cells (Deng et al., 2014a). Here, we showed that percentages of breast cancer stem-like cells (CD44-/CD24+ cells and tumorsphere cells) from HER2 expressing cells were higher than those from MCF7 cells, consistent with the previous report that HER2 signaling positively regulate breast cancer stem/progenitor cells (Geng et al., 2014). We also found that knock-down of ER-α36 expression decreased the populations of the stem/progenitor cells from these HER2-expressing cells, suggesting an important role of ER-α36 in maintenance of breast cancer stem/progenitor cells in these HER2-expressing cells. We further found that in the cells derived from the tumorspheres from these HER2-expressing cells express elevated levels of ER-α36, EGFR and HER2, suggesting there exist the ER-α36-EGFR/HER2 regulatory loops in the ER-positive breast cancer stem/progenitor cells. Again, disruption of these regulatory loops with Lapatinib or Broussoflavonol B was able to sensitize these breast cancer stem/progenitor cells to tamoxifen. Our results thus provided rationales to develop novel therapeutic approaches to treat Her2-expressing breast cancer via eliminating breast cancer stem/progenitor cells by targeting the ER-α36-EGFR/HER2 loops.

In summary, here we provided evidence to demonstrate that there exist ER-α36-EGFR/HER2 positive regulatory loops in HER2-expressing breast cancer cells and that disruption of these regulatory loops restored tamoxifen sensitivity in these cells. Our findings that elevated expression of the ER-α36-EGFR/HER2 regulatory loops is one of the mechanisms by which HER2-expressing and ER-positive breast cancer cells escape the hormonal therapy that was based on estrogen deprivation provided a rational to develop novel therapeutic approaches for antiestrogen resistant patients by targeting these regulatory loops.

Abbreviations

EGFR: Epidermal growth factor receptor; HER2: Human epidermal growth factor receptor 2.

Competing interests

The authors declare that they have no conflicts of interest.

Acknowledgements

This work was supported by Department of Defense grant DAMD 11-1-0497 and Nebraska Tobacco Settlement Biomedical Research Program Awards (LB-595) (Z.Y. Wang). Technical assistance for flow cytometry was provided by Dr. Perry Greg

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Figure legends

Fig.1. HER2 expressing breast cancer cells exhibit enhanced expression of ER-a36 and EGFR as well as tamoxifen resistance. A & B. Western blot analysis of the expression levels of ER−α66, ER-α36, HER2 and EGFR in ER-positive breast cancer MCF7, BT474 and MCF7HER2-18 cells. Cells were treated with indicated concentrations of tamoxifen for seven days and the numbers of survived cells were counted. Each point represents the means of three experiments.

Fig. 2. Knock-down of ER-α36 expression sensitizes HER2 expressing cells to tamoxifen. A. Western blot analysis of the expression levels of ER−α66, ER-α36, HER2 and EGFR in MCF7/HER2-18 cells transfected with an empty expression vector (MCF7/HER2-18/V) and with the ER-α36 shRNA expression vector (MCF7/HER2-18/Si36). B. Western blot analysis of the expression levels of ER−α66, ER-α36, HER2 and EGFR in BT474 cells transfected with an empty expression vector (BT474/V) and with the ER-α36 shRNA expression vector (BT474/Si36). C & D. Cells were treated with indicated concentrations of tamoxifen for seven days and the numbers of survived cells were counted. The columns represent the means of three experiments; bars, SE. * and #, $P<0.05$ for control cells transfected with the empty vector vs the cells transfected with ER-α36 the shRNA expression vector, respectively.

Fig. 3. Dual kinase inhibitor Lapatinib downregulates ER-α36 expression and sensitizes HER2 expressing cells to tamoxifen. A & C. Western blot analysis of the expression of ER-α36 and ER-α66 as well as EGFR and HER2 in parental MCF7, MCF7/HER2-18 and BT474 cells treated with vehicle or indicated concentrations of Lapatinib (LAP) for 12 hours. B & D. Cells were treated with indicated concentrations of tamoxifen (TAM) together with vehicle or 1 μM of Lapatinib (LAP) for seven days and the numbers of survived cells were counted. The columns represent the means of three experiments; bars, SE. * and #, $P<0.05$ for cells treated with vehicle vs cells treated with 0.5 and 1 μM of tamoxifen, respectively.

Fig. 4. ER-α36 disruptor Broussoflavonol B restores tamoxifen sensitivity in HER2 expressing cells. A & C. Western blot analysis of the expression of ER-α36, ER-α66, EGFR and HER2 in MCF7 and MCF7/HER2-18 and BT474 cells treated with indicated concentrations of Broussoflavonol B (BB) for 12 hours. B & D. Cells were treated with indicated concentrations of tamoxifen (TAM) together with vehicle or 1 μM of Broussoflavonol B (BB) for seven days and the numbers of survived cells were counted. The columns represent the means of three experiments; bars, SE. * and #, $P<0.05$ for cells treated with vehicle vs cells treated with 1 μM of tamoxifen.

Fig. 5. Knock-down of ER-α36 reduces populations of the breast cancer stem/progenitor cells from HER2

expressing cells. A & B. Knock-down of ER-α36 expression reduces the populations of CD44+/CD24- cells in BT474 and MCF/HER2-18 cells. The columns represent the means of three experiments; bars, SE. *, P<0.05 for cells transfected with the empty expression vector vs cells transfected with the ER-α36 shRNA expression vector. C. Tumorsphere formation assay was used to assess the effects of ER-a36 knock-down on breast cancer stem/progenitor cells derived from MCF7, BT474 and MCF7/HER2-18 cells transfected with the empty expression vector (BT474/V and MCF7/HER2-18/V), or the ER-α36 shRNA expression vector (BT474/Si36 and MCF7/HER2-18/Si36). The representative results are shown. D. The numbers of tumorspheres formed by these cells. C. The number of cells from dissociated tumorspheres formed by these cells. The columns represent the means of three experiments; bars, SE. *, P<0.05 for cells transfected with the empty expression vector vs cells transfected with the ER-α36 shRNA expression vector.

Fig. 6. Disruption of the ER-α36-EGFR/HER2 positive regulatory loops sensitizes HER2 expressing breast cancer stem/progenitor cells to tamoxifen. A. Western blot analysis of the expression of ER-α36, ER-α66, EGFR and HER2 in the monolayer cells grown on attachment dishes (A) and tumorsphere cells grown on low-attachment dishes (T). Band density (% of β-actin) is also shown. B & C. Tumorsphere cells derived from BT474 and MCF7/HER2-18 cells were treated with 5 μM of Broussoflavonol B (BB) or Lapatinib (LAP) for five days. Western blot analysis of expression levels different proteins was performed. D. Tumorsphere formation assay was used to assess the effects of tamoxifen alone or together with Lapatinib (LAP) or Broussoflavonol B (BB) on the breast cancer stem/progenitor cells derived from MCF7/HER2-18 cells. The representative results are shown. E. The numbers of tumorspheres formed by the cells treated with tamoxifen alone or together with Lapatinib (LAP) or Broussoflavonol B (BB). F. Tumorsphere formation assay was used to assess the effects of tamoxifen alone or together with Lapatinib (LAP) or Broussoflavonol B (BB) on the breast cancer stem/progenitor cells derived from BT474 cells. The representative results are shown. G. The numbers of tumorspheres formed by the cells treated with tamoxifen alone or together with Lapatinib (LAP) or Broussoflavonol B (BB). The columns represent the means of three experiments; bars, SE. * & #, P<0.05 for cells treated with vehicle vs cells treated with 0.5 and 1 μM of tamoxifen.

Supplemental Figure S1. Lapatinib treatment inhibits phosphorylation of HER2 and EGFR in HER2 expressing cells. Western blot analysis of the expression of phosphorylated EGFR and HER2 in MCF7/HER2-18 and BT474 cells treated with indicated concentrations of Lapatinib for 12 hours.

Supplement Figure S2. Proteasome inhibitor MG132 restores HER2 expression in MCF/HER2-18 cells with knocked-down levels of ER-α36 expression. Western blot analysis of the expression of HER2 in MCF7/HER2-18 cells treated with 2 μM of MG132 for 12 hours.

Fig. 1

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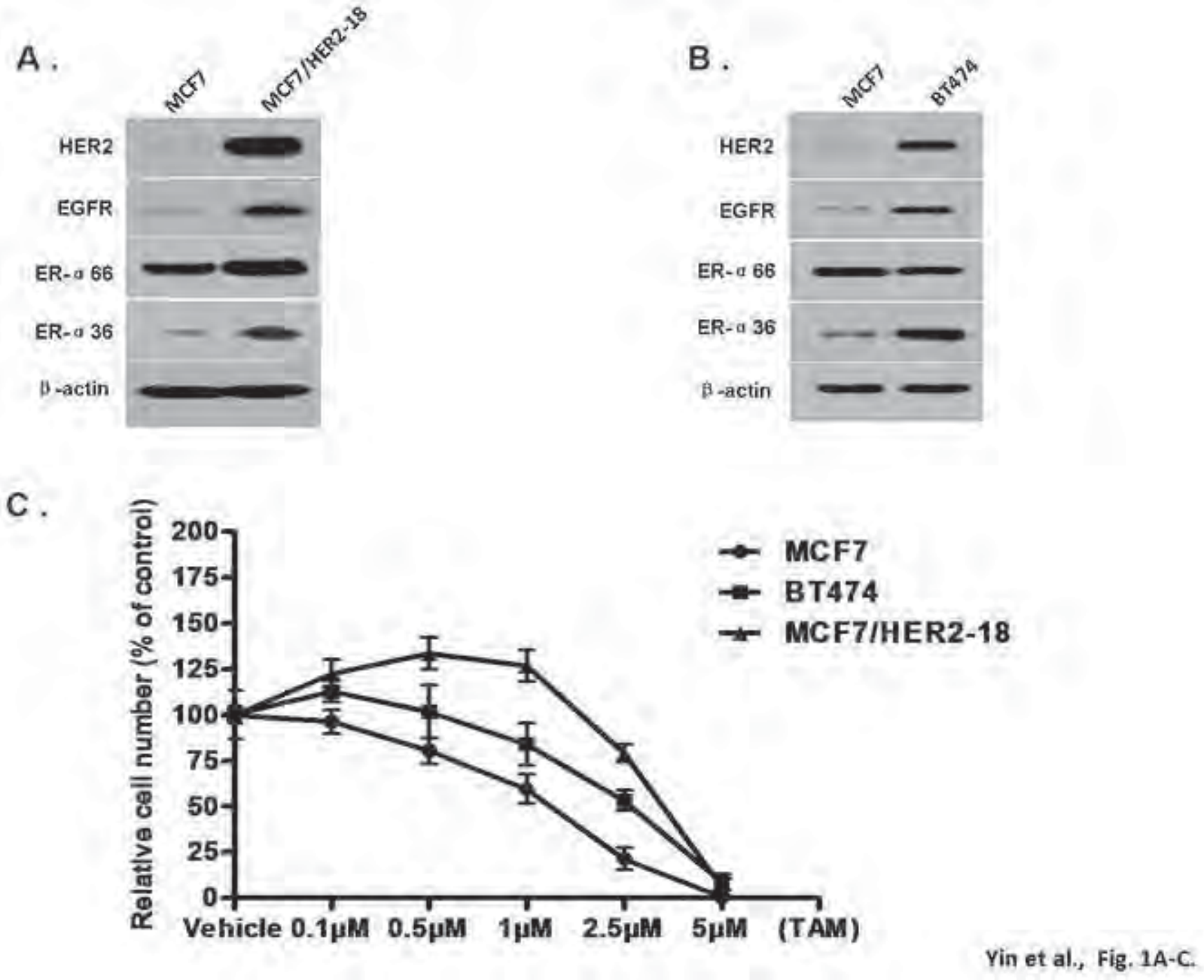

Yin et al., Fig. 1A-C.

Fig. 2

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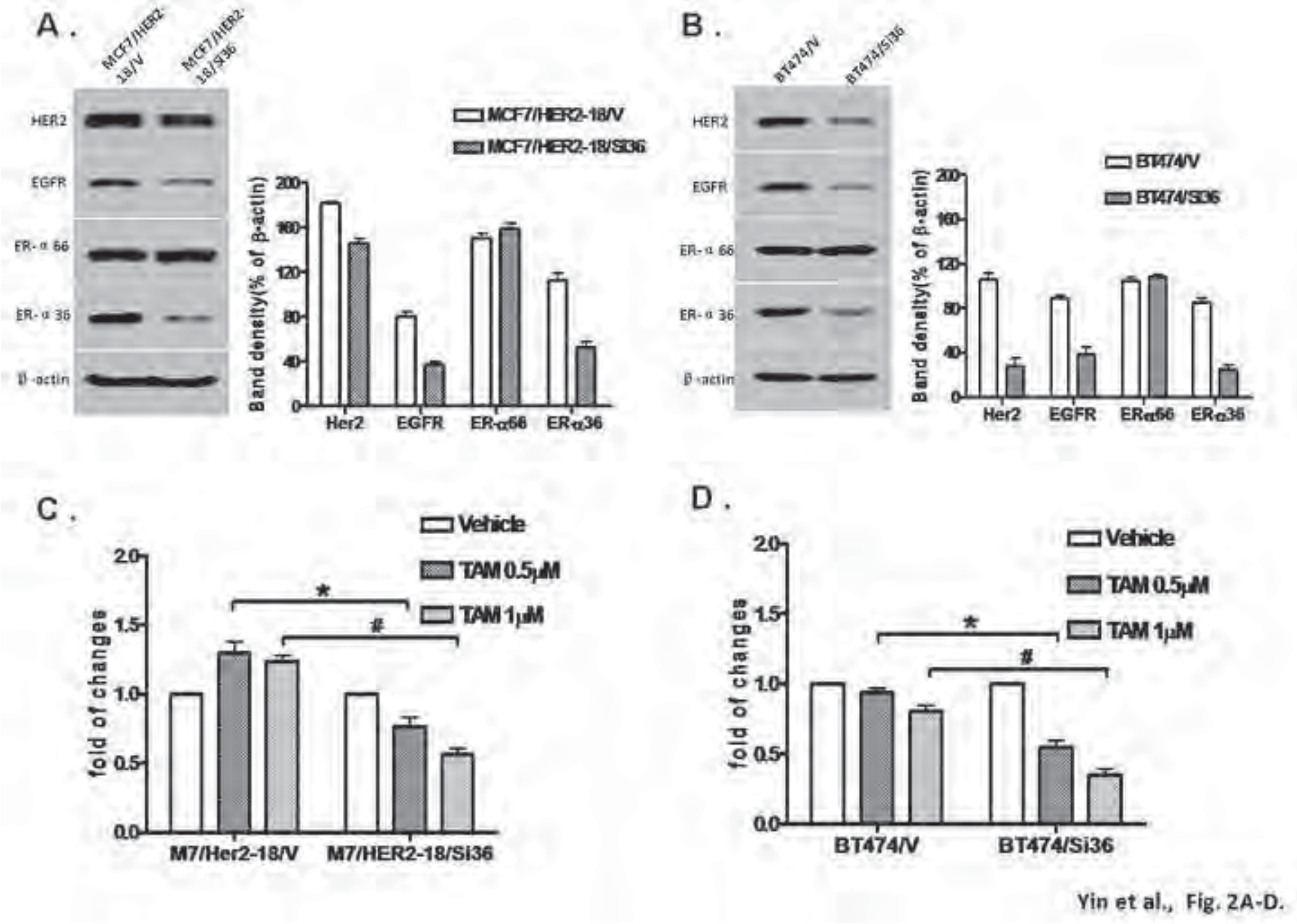

Yin et al., Fig. 2A-D.

Fig. 3

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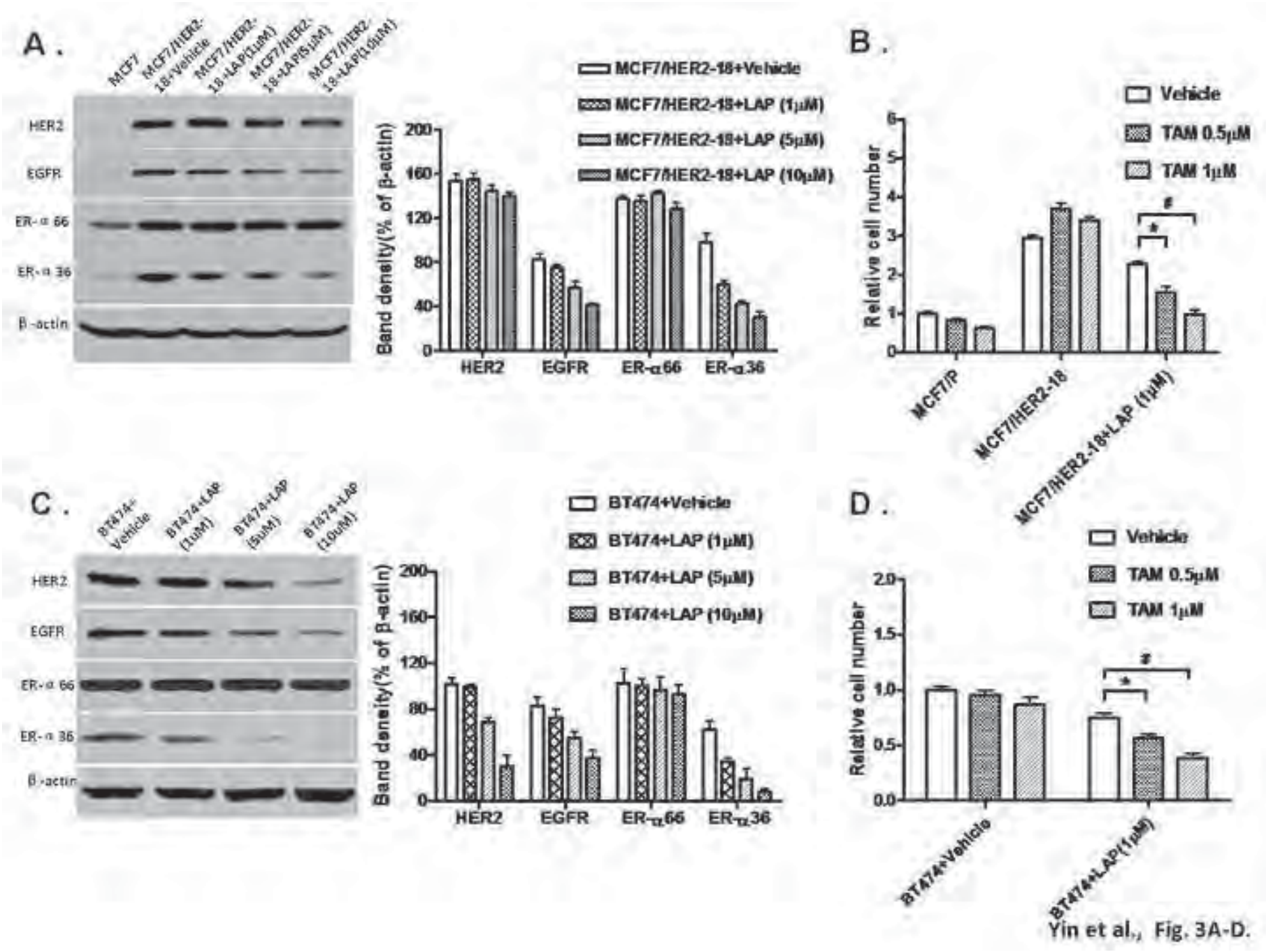

Fig. 4

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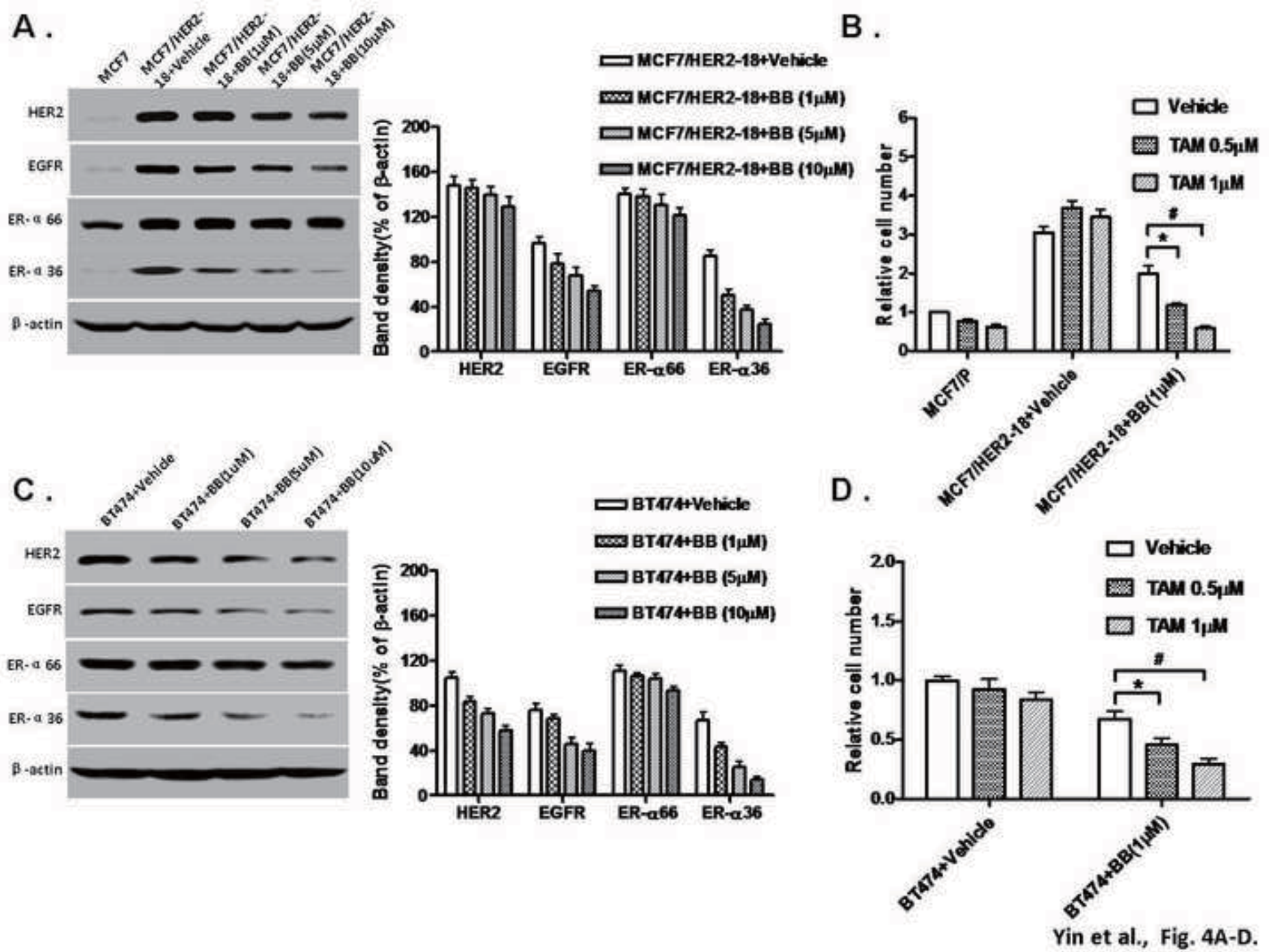

Yin et al., Fig. 4A-D.

Fig. 5A-B

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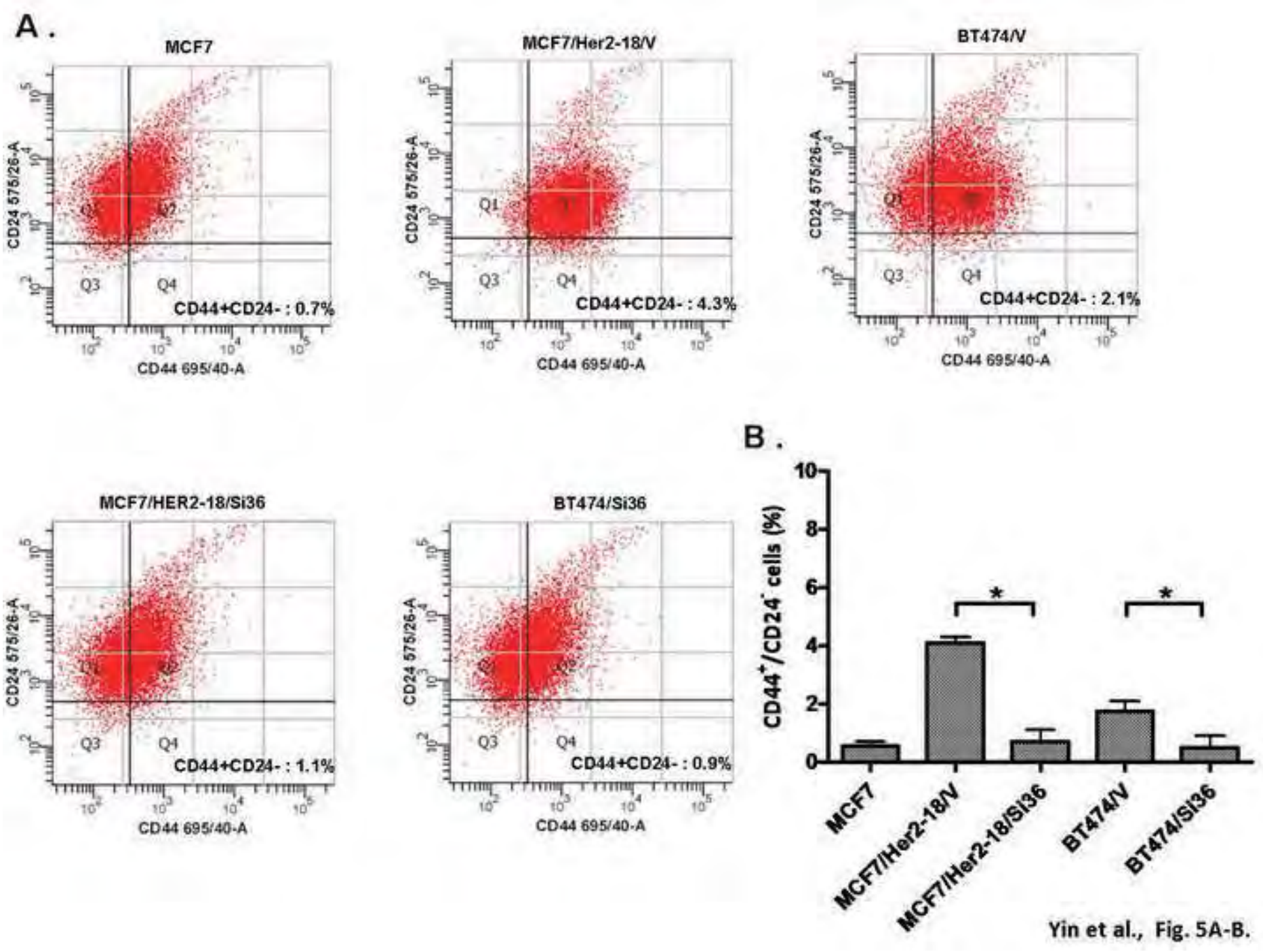

Fig. 5C-E

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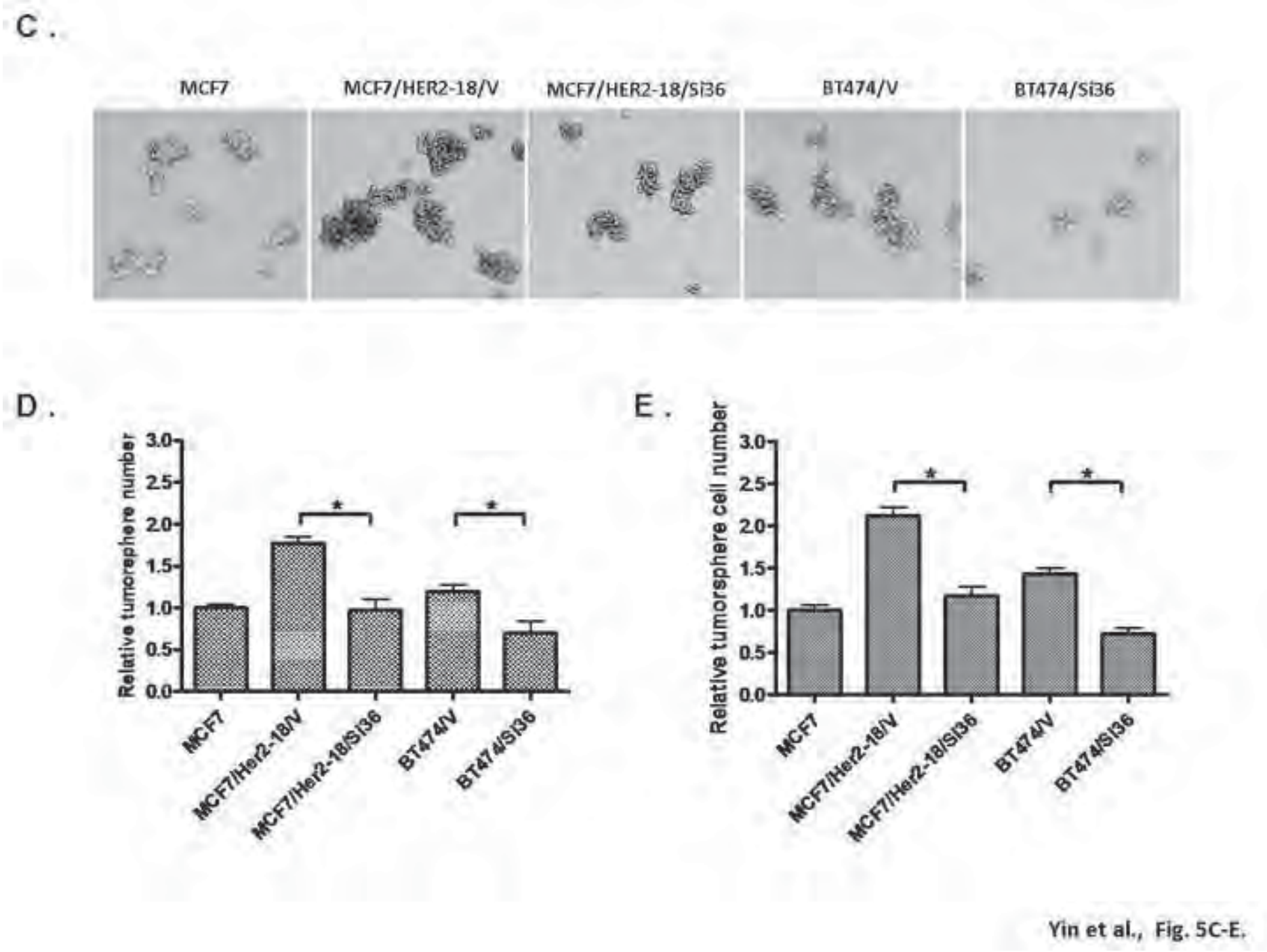

Yin et al., Fig. 5C-E.

Fig. 6A-C

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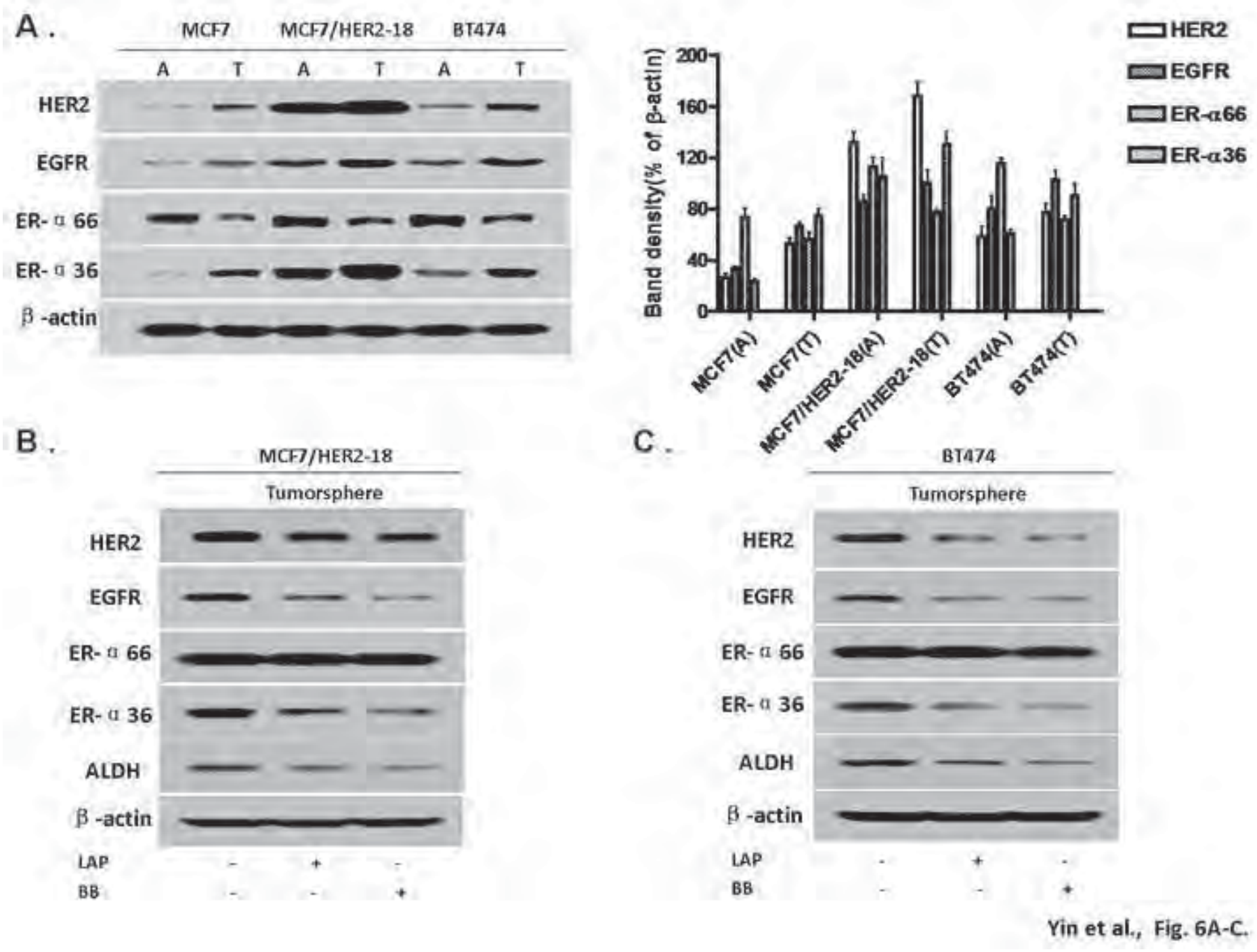

Yin et al., Fig. 6A-C.

Fig. 6D-G

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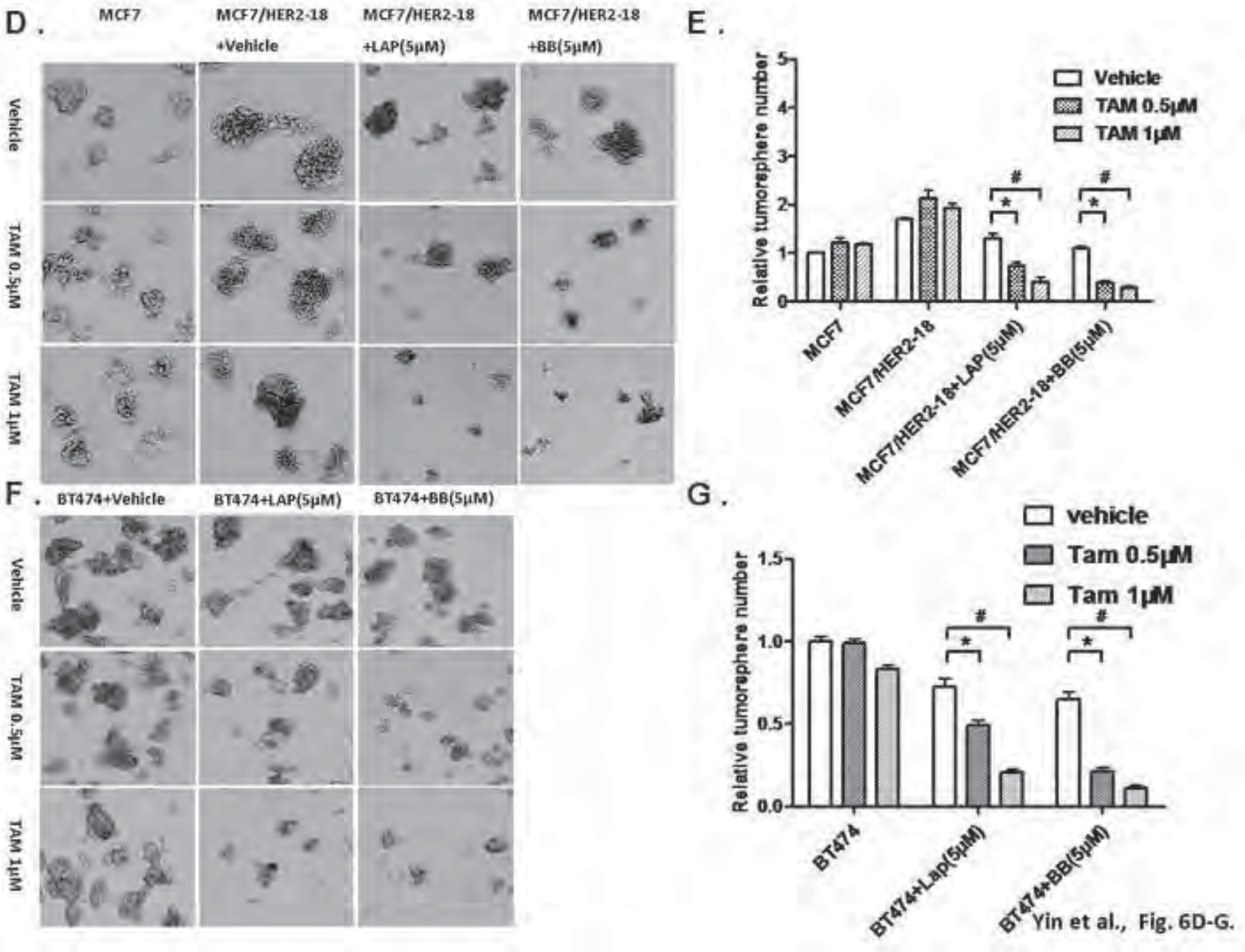

Fig. S1

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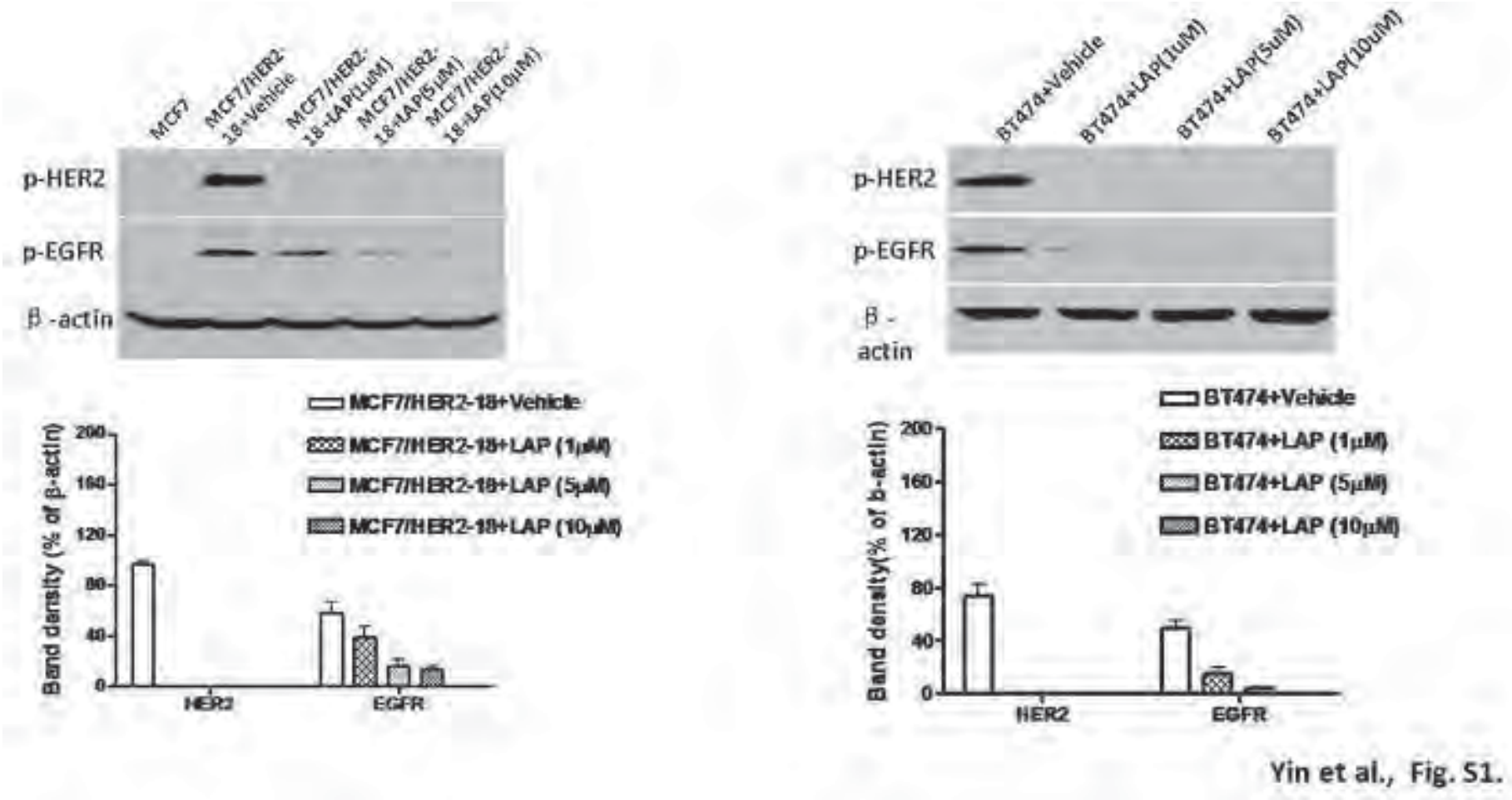

Yin et al., Fig. S1.

Fig. S2

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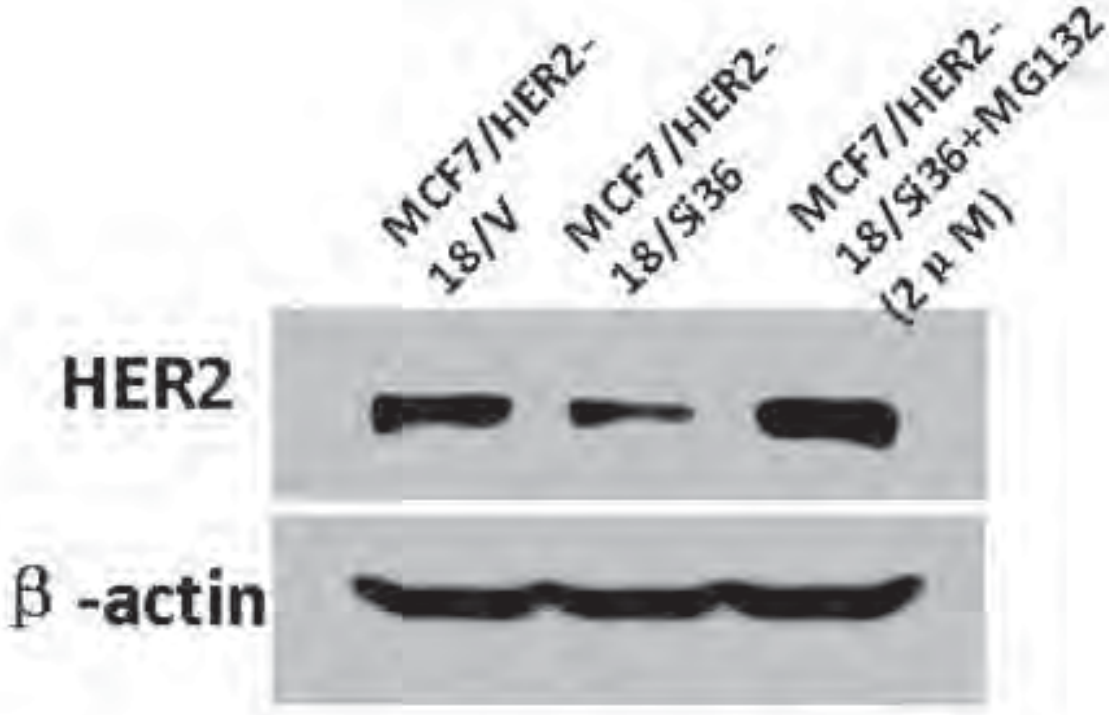

Yin et al., Fig. S2.

Fig. S3

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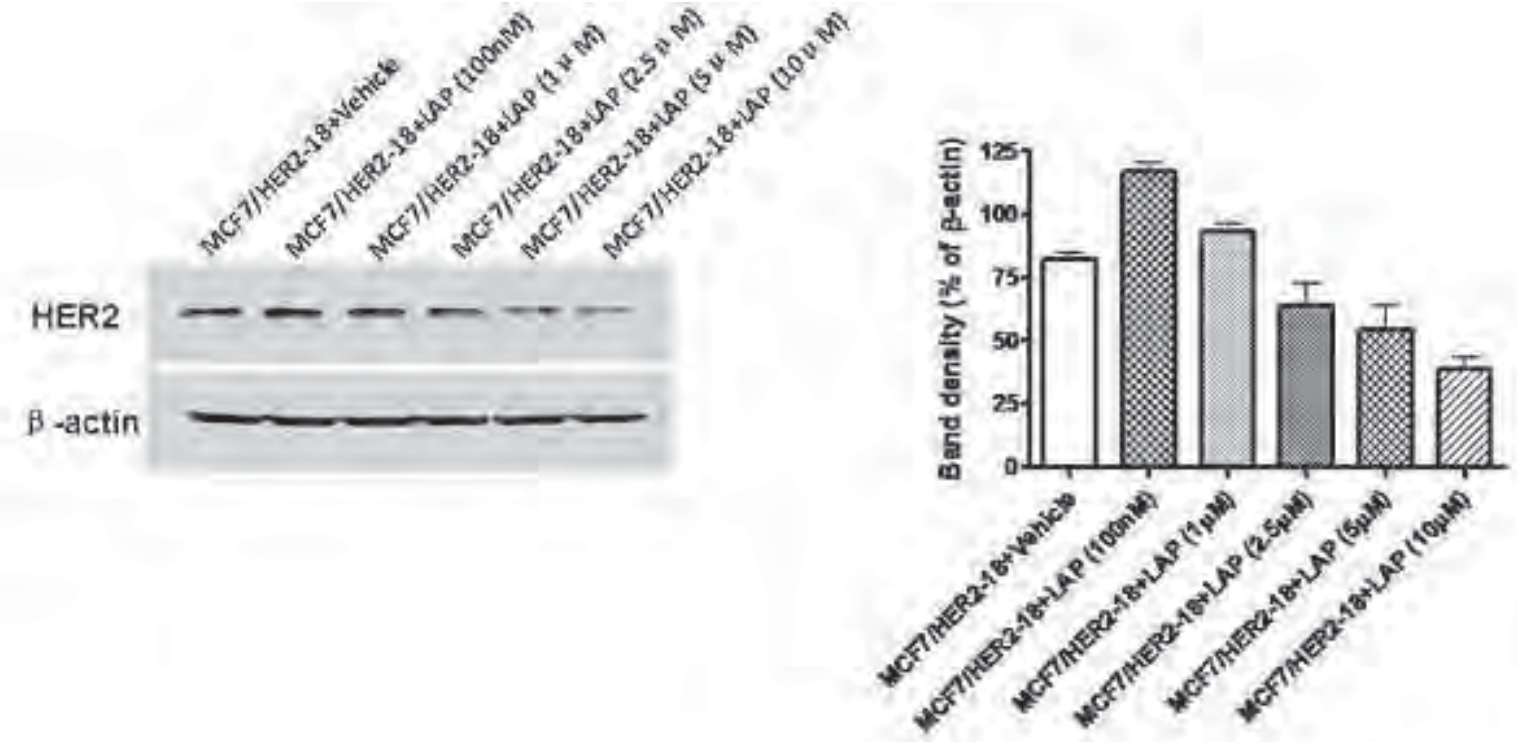

Yin et al., Fig. S3.